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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, A01H 5/00 | A1

(11) International Publication Number: WO 99/10513

(43) International Publication Date: 4 March 1999 (04.03.99)

(21) International Application Number: PCT/IB98/01199

(22) International Filing Date: 5 August 1998 (05.08.98)

(30) Priority Data:

| | | |
|---|---|---|
| 60/055,314 | 7 August 1997 (07.08.97) | US |
| 60/079,042 | 23 March 1998 (23.03.98) | US |
| 09/079,640 | 15 May 1998 (15.05.98) | US |

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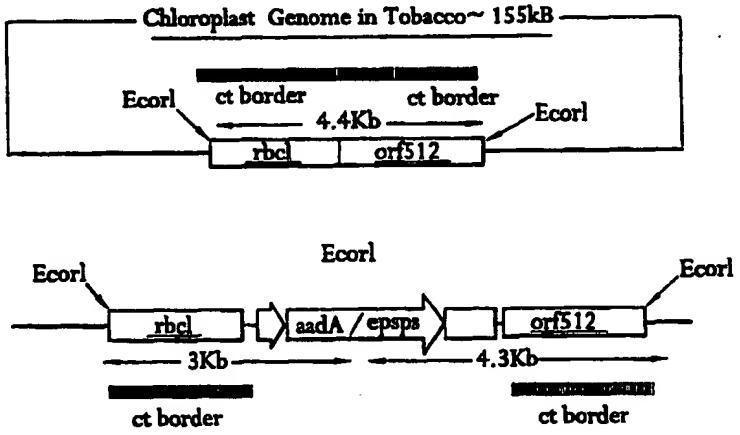
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published**

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: UNIVERSAL CHLOROPLAST INTEGRATION AND EXPRESSION VECTORS, TRANSFORMED PLANTS AND PRODUCTS THEREOF




(57) Abstract

The invention provides universal chloroplast integration and expression vectors which are competent to stably transform and integrate genes of interest into chloroplast genome of multiple species of plants. Transformed plants and their progeny are provided. Monocotyledonous and dicotyledonous plants are transformed which have never been transformed heretofore. Plants transformed with a synthetic gene express valuable biodegradable protein–based polymers (PBPs). Transformed plants produce high value molecules. Resistance is provided to agricultural crops against the major classes of chemical herbicides. Herbicide resistance is used as a lethal selectable marker for chloroplast transformation. The transformed plants are capable of expressing in addition to the targeted trait, a desirable, secondary non–targeted trait. Insect resistance is provided to transformed plants, both against insects that are susceptible to Bt toxins and against insects that have developed resistance to Bt toxins.

# UNIVERSAL CHLOROPLAST INTEGRATION AND EXPRESSION VECTORS, TRANSFORMED PLANTS AND PRODUCTS THEREOF

This application claims the benefit of pending provisional application Serial No. 60/055,314, filed August 7, 1997, which is incorporated herein by reference in its entirety. This application also claims the benefit of pending provisional application Serial No. 60/079,042, filed March 23, 1998, entitled "Universal Chloroplast Integration and Expression Vector, Transformed Plants and Products Thereof", which is incorporated herein by reference in its entirety. Further, this application is a continuation-in-part of pending patent application Serial No. 08/591,407, filed January 25, 1996, by Henry Daniell, which is a continuation of application Serial No. 08/215,020, filed March 18, 1994, now abandoned, which in turn was a continuation of application Serial No. 07/249,616, filed September 26, 1988, now abandoned.

## FIELD OF THE INVENTION

This application pertains to the field of genetic engineering of plant genomes, particularly the genetic engineering of the genome of plant plastids, such as chloroplasts and to the stable transformation of chloroplast genome of any plant species.

## RELATED CASES

This application relates in particular to a universal chloroplast expression and integration vector which is competent to transform any plant with one or more genes of interest. The earlier patent application Serial No. 08/591,407, teaches plant cells transformed by means of an expression cassette comprising an exogenous DNA sequence which is stably integrated (covalently linked) to the chloroplast genome of the cell of a target plant. "Stably" integrated DNA sequences are those which are inherited through genome replication by daughter cells or organisms. This stability is exhibited by the ability to establish permanent cell lines,

al., 1990). In the southern US, red rice has become a major weed because herbicides that kill it also kills cultivated rice. Decreased prices are paid for cultivated rice contaminated with red rice. Some researchers have introduced the *bar* gene conferring resistance to glufosinate (Liberty) into cultivated rice to combat this weed (Oard et al., 1996; Sankula et al., 1996). However, due to sexual compatibility, introduction of a nuclear-expressed gene will allow transmission of that resistance trait into red rice via pollen.

Similarly, transgenic oil seed rape, genetically engineered for herbicide resistance outcrossed with a weedy relative, *Brassica campestris* (field mustard) and conferred herbicide resistance even in the first back-cross generation under field conditions. (Mikkelson, T. R., et al., 1996).

Maternal inheritance of introduced genes prevents gene escape through pollen. Engineering foreign genes through chloroplast genomes (which are maternally inherited for most of the crops) is a solution to this problem. Also, the target enzymes or proteins for most herbicides (e.g. amino acid/fatty acid biosynthetic pathways or photosynthesis) are compartmentalized within the chloroplast. Another important advantage of chloroplast transformation is the higher levels of foreign gene expression due to a very high copy number (5000-10,000) of chloroplast genomes in plant cells. Because the transcriptional and translational machinery of the chloroplast is prokaryotic in nature, herbicide resistant genes of bacterial origin can be expressed at extraordinarily high levels in chloroplasts.

**Transformation of the Chloroplast Genome.** Early investigations on chloroplast transformation focused on the development of in organello systems using intact chloroplasts capable of efficient and prolonged transcription and translation (Daniell and Rebeiz, 1982; Daniell et al., 1983)

amenable to plastid transformation due to inherent inefficiencies within those systems. Also, sequential/serial selections (repeated selections), deemed important for achieving homoplasmy (Daniell, 1997), may not be feasible using those regeneration systems employed. Recent development of unique corn (Rudraswamy, 1997) and rice (unpublished) transformation/regeneration protocols have the potential to exhibit substantially increased efficiencies and allow more than one round of selection during regeneration.

Maliga et al. in U.S. patent 5,451,513 and Svab et al., 1990 propose a transformation of the plastid genome of tobacco by a non-lethal selection technique which employs plastid DNA encoding a non-lethal selectable phenotype. According to Maliga et al. a non-lethal selection is absolutely essential for obtaining transplastgenic lines.

Unlike the Maliga et al. technique, the method of the invention provides a selection which is lethal to all non-transformed plants, but for tobacco. Only the transformed plants survive and continue to grow. This lethal selection takes place with virtually all antibiotics, including spectinomycin and streptomycin in a medium containing the antibiotic in a concentration of 500 - 1,000 μg/ml. Similar conditions were shown to be non-lethal for tobacco by Maliga et al. Moreover, unlike the technique of Maliga et al., in accordance with the invention, transformation to homoplasmy can be achieved even in the first round of selection.

In European Patent Application No. 0 251 654, Cannon et al. describe transposon-mediated chloroplast transformation of tobacco for instance, using the bacterial transposon Tn5. The vector containing the transposon is targeted at a chromosomal region known to be a "transcriptionally silent" region in order to preserve the transcriptional integrity of the native genes. Such a transcriptionally silent region is identified to be located between two known divergent promoters of chloroplast genes, e.g. the promoters for the genes for the chloroplast large subunit of ribulose bisphosphate carboxylate

coding sequence and eliminate the need to construct vectors which each one is specifically suited to transform the chloroplast genome of the particular plant species which is to be transformed.

The problem to construct such a universal vector competent to transform different plants has to the knowledge of the inventor, not yet been solved.

**Prior Art Concepts of the Intergenic Spacer Region.** While the nucleotide sequence of coding regions of the genome, including the chloroplast genome, are often conserved between species, in contrast the sequences flanking functional genes, i.e. the spacer regions between coding regions typically are not conserved. The accepted dogma for lack of conservation, and thus the low degree of homology between species of spacer regions, is that the spacer regions typically do not perform essential functions. Therefore, there is little, if any, selective pressure to conserve the sequence of spacer regions between species. The sequence of the spacer regions may be altered without undesirable effects.

Stummann et al., 1988, disclose that the gene order of the ribosomal RNA operon of the chloroplast genome is the same between different species of plants, including tobacco, maize, and a liverwort, *Marchantia*, and that the coding sequences of this operon are highly homologous. Stummann also discloses that the interspecies homology of the operon is less than the interspecies homology of the gene coding regions. This is consistent with the lack of conservation of spacer regions; and suggests that the interspecies homology of spacer regions in the ribosomal RNA operon is relatively low.

The invention, contrary to the dogma of lack of conservation of the spacer regions, uses spacer regions that are highly conserved between different plants to construct vectors competent to transform a variety of plants.

**The Universal Vector.** The invention has several useful embodiments. The invention provides a universal integration and expression vector hereinafter referred to as "UV" and its use for the expression of at least one phenotype in a variety of different plants.

The integration expression universal vector of the invention comprises an expression cassette (further described below) which comprises the necessary genetic elements to transiently or preferably stably transform the plastids e.g. chloroplast genome of a target plant cell with a foreign (heterologous) DNA coding for a molecule of interest, like a phenotype to be expressed by the plant or a non-plant high value molecule, like a biologically active peptide (or polypeptide). The universal vector is constructed with a transcriptionally active region of a chloroplast genome that is highly conserved in a broad range of chloroplast genomes of higher plants. Preferably that region is the spacer 2 region; the intergenic spacer region between the t-RNA$^{Ile}$ and the tRNA$^{Ala}$ region. Such region is often referred to herein as a "spacer" region because in the chloroplast genome it is intergenic between several genes in the rRNA operon which is transcribed by one promoter. When built into the universal vector such region is generally referred to herein as a "border" or preferably as a "flanking sequence" or "flanking sequences". This is because in the universal vector, the operably joined genetic elements for transforming stably the plastid of the target plant are flanked on each side by a sequence i.e. a fragment of the spacer region. The flanking sequences in the vector and the spacer sequences in the chloroplast genome have sufficient homology to each other to undergo homologous recombination. The universal vector is inserted into the spacer of a transcriptionally active region in the chloroplast genome. Generally, the spacer region is positioned in the inverted repeat region of the chloroplast genome. The rest of the construct, i.e. other than the flanking sequences and the expression cassette, is generally

serve as a marker associated with the expression cassette or with the universal integration expression vector. This facilitates identification of the plant cells in which the foreign gene has been stably integrated. Marker genes are known in the literature, for instance β-lactanase, herbicide resistant genes such as the mutant *psb*A gene or EPSPS-*aro*A, the cat gene which encodes chloramphenicol acetotranferase, and the uidA gene encodes β-glucuronidase (gus)and others.

It is recognized that tobacco is unique in being not susceptible to the lethal affect of streptomycin and spectinomycin. Though tobacco leaves lack the pigmentation when exposed to a medium with such an antibiotic, continued growth is observable. However, this property of tobacco is readily circumvented. There are numerous antibiotics available which are lethal for tobacco, like hygromycin. Another approach is to select a gene which expresses a visible marker like a color, fluorescence, etc., like the reporter gene mGFP, that codes for a green fluorescent protein.

**Method of Transformation.** The invention provides a transformation method which can produce homoplasmy (integration of foreign genes into all of the chloroplast genomes of the plant cell) after a first round of selection without the need for a further selection process. The method for transforming a plant uses the universal vector constructed with flanking sequences from a plant species other than the species of the target plant to be transformed. Alternatively, the vector may contain flanking sequences from the same plant species as the target plant, including from tobacco.

**Method to Construct the Universal Vector.** The invention further provides a method to construct the universal chloroplast integration and expression vector. To this effect, a spacer portion of the chloroplast genome from any plant is determined to be highly homologous to more than one

in this context also includes portions of plants such as explants like cuttings, tissue cultures, cell suspensions, and calli.

Thus, the invention includes the stably transformed multicellular plants, their progeny, the seed, and the transformed plastids, e.g. the chloroplast, etc., and method of regenerating the transformed plants.

In this specification and in the claims, when reference is made to different "species", the term "species" refers not only to "species" but to varieties within a species, genera, families, order, and divisions of the plant kingdom. Thus, a universal vector which can be used to transform plants of different species is understood to be able to transform plants of different varieties within a species, different genera, different families, different orders, and different divisions. The term "plant" (or "plants") is intended to be generic as used herein.

## Expression of Non-Plant Products

**Biopolymer genes.** Another embodiment of the invention using the universal integration and expression vector provides plants transformed with a synthetic biopolymer gene that codes for biodegradable protein-based polymers (PBPs).

These polymers have important properties of practical importance, discussed hereinafter.

**Production of High Value Molecules-Biologically Active Molecules.** The intriguing discovery that transformation with a synthetic gene which need not have a natural analogue in plant or animal, to produce PBPs, is feasible, has shown the wide applicability of the vector in yet another field of human endeavor: the production of biologically active molecules, like pharmaceuticals in plants, from any gene or functional fraction thereof, synthetic or natural.

cassette, preferably by means of the universal vector, to cause it to produce a non-targeted (secondary or other) trait (or phenotype). [See, for example, Penazloza, V., et al. (1995), who report that expression by gromycin β-phosphotransferace gene confers resistance to the herbicide glyphosate.]

In another aspect of the invention, herbicide tolerance is used as a marker gene for chloroplast transformation.

**Insect Resistance.** A further embodiment of the invention provides insect resistance. With the increased concerns of using chemical pesticides, the use of *Bacillus thuringiensis* (Bt) formulations has been widely advocated. *Bacillus thuringiensis* produces many types of crystalline inclusions which are toxic to insects. The proteins comprising these inclusions have been categorized based on insecticidal host range, and protein homology. The CRYI and CRYII toxins have insecticidal activity against lepidoptera, or lepidoptera and diptera, respectivly. CRYI protoxins are 130-135 kDa in size which are enzymatically cleaved into proteins of 65kDa for insecticidal activity.CRYII protoxin is 65kDa in size with a protein with a molecular mass of 60-62 kDa for insectcidal activity. Many commercially important insects pests (especially in the family Pyralidae) are susceptible to CryIIA toxin, including European corn borer, *Ostrinia nubilalis*, lesser cornstalk borer, *Elasmopalpus lignosellus*, cowpea pod borer, *Maruca testulalis*, tobacco budworm, *Heliothis virescens*, tobacco hornworm, *Manduca sexta* and gypsy moth *Lymantria dispar*, Daniell et al. 1994.

However , Bt formulations have not been as effective as anticipated primarily due to their susceptibility to UV radiation, inadequate coverage, expense and limited host range. Delivery of Bt toxins via Bt-transgenic plants is therefore appealing.

## DESCRIPTION OF THE DRAWINGS

Figure 1 shows a map of the tobacco chloroplast genome. The thick lines in the genome map represent the inverted repeat regions of the chloroplast genome. The arrows labeled "UV" represent the insertion sequences for the preferred embodiment of the universal integration and expression vector (UV); the arrow labelled "TV" represents the insertion sequence for the tobacco vector (TV).

Figure 2A shows the tobacco chloroplast vector (TV) pZS-RD-EPSPS for expression of herbicide resistance.

Figure 2B shows the universal chloroplast expression and integration vector (UV), pSBL-RD-EPSPS for expression of herbicide resistance.

Figure 3A shows the universal chloroplast integration and expression vector, pSBL-CG-EG121 for biopolymer expression.

Figure 3B shows the tobacco integration and expression and vector, pZS-CG-EG121 for biopolymer expression.

Figure 4A-4E shows the sequence homology of spacer regions between tobacco and other crop species .A site for foreign gene insertion is shown by arrows. Upstream of the site for foreign gene insertion the site of an origin of replication (*ori*) is shown.

Figure 4F-4G shows the sequence alignment of the spacer (64 bp) region 16S-23S rDNA from several crop species with the tobacco chloroplast sequence where (+) represents the positive and (-) the negative strands, respectively.

Figures 5A-C shows construction of the vector pSBL-Ct border.

Figures 6A-C shows construction of the vector pSBL-CtV1 selectable marker gene cassette containing a chloroplast 16S rRNA promoter (Prrn), the *aad*A gene and a 3' untranslated region of the chloroplast *psb*A gene.

Figures 7A-7D shows vectors pSBL-CtV2, pSBL-CtV3, pSBL-CtVH, pSBL-CtVHF, respectively.

tobacco vector (TV). Probes were from chloroplast border sequences (A) or from polymer (EG121) gene sequences (B)

Figure 20 shows Southern blot analysis performed with the transformants from PBP transgenic plants using the universal vector (UV). Probes were from chloroplast border sequences (A) or from the aadA gene (B).

Figure 21A shows foreign gene transcript levels analyzed by northern blotting using total RNA isolated from the control, chloroplast transformants and a nuclear tobacco transgenic plant highly expressing the synthetic biopolymer gene (EG121).

Figure 21B shows an enlargement of lanes 4-7 of Figure 21A.

Figure 22 shows Western blot analysis of purified polymer protein from transgenic plants.

Figure 23A shows the higher growth rate of *E. coli* containing the tobacco vector with the EPSPS gene.

Figure 23B shows the higher growth rate of *E. coli* containing the universal vector with the EPSPS gene.

Figures 24A-24B show the integration of foreign genes into the plastid genome by PCR using *rbcL* and *aadA* primers (A), or 16SRNA and *aadA* primers (B).

Figures 25A-25C show the integration of the aroA gene into the chloroplast by Southern analysis and the high generation of homoplasmy using EPSPS probe (A) or *rcb*L-*orf*512 probe. The site of integration is shown in (C).

Figures 26A and 26B show generation of seeds collected from control and transformed tobacco plants, respectively, in the presence of the selectable markers.

Figures 27A and 27B show transgenic and control tobacco plants sprayed with glyphosate.

Figures 28A and 28B show tobacco susceptibility (control) and resistance (transformed) to insects.

Figure 29 shows (Western blot analysis) total protein isolated from control and transgenic tobacco plants.

If longer border sequences which include non-homologous portions, are incorporated into the vector, the non-homologous portion of the sequence will be "looped out" and "clipped off", in the recombination process and will not integrate into the target chloroplast genome.

Different universal vectors can be constructed with the spacer region. For instance, shorter or longer flanking sequences can be constituted with part or all of the *trnA* and *trnI* genes adjacent to `spa 2'.

A preferred universal vector comprises the flanking sequences and an expression cassette which comprises the following genetic elements to provide for transcription and translation of the DNA coding sequence organized in the following order (from the 5' to the 3' ends): a 5' part of the flanking sequence, a promoter functional in chloroplast, a DNA sequence with appropriate cloning site(s) for insertion of one or more coding sequence(s) for the desired phenotype or molecule of interest, and for a selectable marker, a terminator of transcription and a 3' part of the flanking sequence. The order of the DNA sequences coding the desired phenotype and the selectable marker can be switched. Additional flanking plant DNA sequences can be provided to promote stable integration. Preferably, the flanking sequence comprises an origin of replication (*ori*).

In a particular illustration, the highly conserved spacer region resides in the inverted repeat of the chloroplast genome. However, the particular location of the spacer in the chloroplast genome is not as important as its high homology with the spacer region of different plants.

Further, as may be seen in Figures 4F-4G, the spacer 2 (or spa 2) sequence which is 64bp long is too short to include the chloroplast genome *ori* which resides upstream, of that spacer. If it is desired to include the *ori*, a longer spacer sequence encompassing the *ori* will be selected which will include the spacer sequence and an additional sequence in the flanking sequences. This will provide a longer

the vector pSBL-CtV1, the selectable marker gene cassette was inserted into the *trnI* gene (Figure 6A). In the vector pSBL-CtV2 (Figure 7A), the selectable marker gene cassette was inserted between the *trnI* and *trnA* genes in the spacer region, in the direction of the 16S rDNA transcription. In the vector pSBL-CtV2 R (map not shown), the selectable marker gene cassette was inserted between the *trnI* and *trnA* genes in the spacer region, in the direction opposite of the 16S rDNA transcription.

Several genes of interest have been inserted into the pSBL-CtV2 vector, a preferred embodiment of the universal vector. For example, the vector pSBL-CtV3 (Figure 7B) contains the reporter gene mGFP that codes for a green fluorescent protein, isolated from jelly fish. This gene may also be useful for visible selection of transformed plants or in ornamental horticulture, for example, in ornamental crops like Christmas trees or even lawn grass, which may glow with green fluorescence upon illumination with blue light.

The vector pSBL-CtVH (Figure 7C) contains a different selectable marker, hygromycin phosphotransferase (hph gene driven by the chloroplast atpB gene promoter), which confers resistance to the antibiotic hygromycin. This vector may be used to transform plants that are resistant to other antibiotics and is particularly useful for transforming monocots, which are generally resistant to other commonly used antibiotics. This gene may confer additional traits such as herbicide resistance, a non-targeted trait.

Vector pSBL-CtVHF (Figure 7) contains the GFP and hph genes, which can be used for lethal or a combination of lethal/visible selection.

**A Chloroplast Vector Specific for Tobacco and a Universal Chloroplast Vector.** The tobacco chloroplast vector pZS-RD-EPSPS (Figure 2A) ("TV") and the universal vector pSBL-RD-EPSPS (Figure 2B) ("UV") contain both the Prrn promoter (of the 16S rRNA), the *aadA* gene (for spectinomycin selection), the mutant

invention also contains the chloroplast origin of replication (*oriA*), as confirmed in several crop species including pea (Nielsen et al., 1993) and tobacco (Lu et al., 1996), which may explain the highly conserved sequence homology in this region. This origin of replication provides increased number of plasmid templates for efficient integration into the recipient chloroplast genome and achieve homoplasmy.

As shown above, in the construction of the universal vector, an expression cassette containing a chloroplast promoter, a selectable maker gene conferring resistance to an antibiotic (or other selected marker), a gene encoding the target molecule, and the other elements (as described herein) are inserted at a convenient restriction site into the DNA fragment containing the spacer region. If desired, the foreign gene encoding the target molecule may be inserted into the expression cassette after insertion of the cassette into the DNA fragment containing the conserved spacer region so that, before insertion, the cassette will include multiple cloning sites for insertion of one or more DNA coding sequences.

The position of the restriction site in the spacer sequence can determine the respective length of the two flanking sequences, which will be fractions (of different or same length) of the spacer region. Thus, the two flanking sequences need not be identical in length as long as each one contains enough of complementarity to the target chloroplast genome to promote homologous recombination.

Because the vector of the invention has such a high degree of homology to the spacer region of the chloroplast genomes of multiple species of plants, it is competent to transform, not only the species of plants from which the border sequence of the vector is derived, but any plant species.

As used in this specification, the term "homologous" means that a DNA sequence from one plant species possesses regions of sequence identity to portions of a DNA sequence from another plant species. That is, if two DNA sequences are

The flanking sequences shown by patent No. 5,693,507 and the other publications to promote stable integration are not the flanking sequences of the universal expression and integration vector described herein which are highly conserved from plant species to plant species, whereas the flanking sequences of that patent and the other publications are not.

**Identification of Intergenic Spacer Sequences.** The invention provides methods to identify appropriate untranscribed intergenic spacer sequences in plants which are appropriate to construct the universal vectors. The method comprises isolating plastid genomic DNA, carrying out hybridization with a radioactive labeled probe of a known spacer, detecting and isolating plastid sequences which exhibit the desired degree of homology with the probe. As an illustration, to determine if a plastid genome of unknown structure and sequence possesses the spacer region, Southern blots utilizing the tobacco spacer region as a probe are carried out. Plastid genomic DNA is isolated and cleaved by an appropriate restriction enzyme according to established procedures. Hybridization with the spacer probe is conducted under both stringent (e.g., 50% formamide at 68$^{0}$C, wash in 0.1 X SSC at 68$^{0}$C) and non-stringent condition (e.g., 6 X SSC, at 68$^{0}$C, wash in 2 X SSC at 50$^{0}$C) (1 X SSC is 0.15M NaCl, 0.015M sodium citrate) to detect plastid sequences exhibiting approximately 90-100% homology or 60-100% to the tobacco spacer, respectively. The identified plastid sequences are then isolated. If one's requirement of homologous recombination is more permissive, a lower degree of hybridization to the probe, such about 60% can be satisfactory.

Thus, any known or unknown spacer region of sufficient homology for recombination is suitable for the construction of the UV. Likewise, the known sequence of any intergenic highly conserved spacer sequence may be used to identify and isolate plastid sequences which are homologous to a known spacer sequence.

**Method for Transformation.** The expression cassettes may be transformed into a plant cell of interest by any of a number of known methods. These methods include, for example, the following. Transformation by tungsten particle bombardment , polyethylene-glycol- mediated transformation, use of a laser beam, electroporation, microinjection or any other method capable of introducing DNA into a chloroplast. See, for example, Sanford, 1988; Daniell, 1993; Daniell, 1997; U.S. Patent No. 5,693,507; Kin Ying et al., 1996. The use of these techniques permits the application of the invention described herein to a wide variety of both monocotyledonous and dicotyledonous plants.

## Expression of Non-Plant Molecules From Transformed Plants

The increased usefulness of the universal expression integration vector of the invention is clearly shown by the competency of the vector to generate transformed plants to express non-plant and valuable molecules.

**Biodegradable Protein-Based Polymers.** In accordance with another embodiment of the invention, the universal vector has been used to transform tobacco with a synthetic gene expressing protein-based polymers (PBPs). Such polymers, and the genes expressing them, are known in the literature. (Daniell, et al., 1997). Of particular interest, are protein-based polymers (PBP) which have repeating pentamer sequences like GVGVP (Yeh et al., 1987). These PBP polymers show useful inverse-phase temperature transition property. The protein becomes insoluble when the temperature is raised above the transition state. PBPs offer a wide range of materials similar to that of the petroleum-based polymers, such as hydrogels, elastomers, and plastics. They also show remarkable biocompatability, thereby enabling a whole range of medical applications including the prevention of post-surgical adhesions, tissue reconstruction and programmed drug delivery

be comprised in a universal vector, or, if desired, in an expression cassette, as described above.

Transgenic plants are known to produce valuable biologically active molecules by nuclear transformation but not via chloroplast transformation. See the following literature references, all of which are incorporated by reference. Daniell, 1995; Miele, 1997; Lyons, 1996; Daniell, and Guda, 1997; Arntzen, 1997.

**Expression of Biologically Active Molecules.** Plants transformed in accordance with the invention with the universal vector or with the expression cassette can be made to express valuable biologically active molecules in chloroplast containing parts of the plants. The plants will then be harvested by known practices. The transformed plants containing these products can thus be administered orally. Arntzen, 1997. Production of pharmaceuticals by transgenic plants has been performed with peptides (proteins) for many pharmaceutical applications, including vaccines, immunomodulators, growth factors, hormones, blood proteins, inhibitors, and enzymes. Typical transgenic plant derived biologicals which have been reported, include vaccines against viral diseases; viral peptide epitopes, like human immunodeficiency virus, non-viral peptide epitopes, bacterial antigenic proteins; bioactive peptides, recombinant toxins, plantibodies (recombinant antibodies), serum proteins, and plant secondary metabolites. All these products can be expressed in chloroplasts of transgenic plants in accordance with the invention.

Typical pharmaceutical peptides or proteins produced in transgenic plants include hepatitis B surface antigen, norwalk virus capsid protein, foot-and-mouth disease virus, human rhinovirus 14, human immunodeficiency virus, *S. mutans* surface protein, *E. coli* enterotoxin, B subunit, malarial circumsporozoite epitopes, mouse ZP3 protein epitope (vaccine); mouse catalytic antibody 6D4, mouse mAB Guy's 13, mAB B1-8, anti-phytochrome Fv protein, anti-substance P

the other known methods, and the insulin is collected. Alternatively, insulin polymer fusion product can be expressed in an edible crop or edible parts of the crop.

The technique of fusing a DNA sequence coding for a molecule of biological activity to a synthetic gene expressing a protein-based polymer for expressing, in a suitable bacterial or yeast host or a transformed plant, is a highly promising method of wide applicability.

**Recombinant Human Serum Albumin in Plants.** In nuclear transgenic tobacco and potato plants, recombinant human serum albumin (rHSA) that is indistinguishable from the authentic human protein has been produced (Sijmons et al., 1990). This showed the expression of a valuable protein in transgenic plants, but also that it was possible to achieve proper processing by fusion of HSA to a plant pro-sequence that resulted in cleavage and secretion of the correct protein. The chloroplast genome of a selected plant like tobacco can be readily transformed with a universal vector as described herein and made to express HSA.

**General Applicability.** As described herein the universal vector permits, in accordance with the invention, the transformation of plants, make the plant to express a biological molecule which can impart a desired phenotype to the plant and/or produce a desired product which may, but need not have biological activity (or a precursor to a final product). The coding nucleotide sequence can be synthetic, or natural. The produced molecule can be foreign to the plants, non-functional in the plant or functional. The universal vector has broad applications in the domain of plant transformation.

It is contemplated that any biologically active molecule (precursor or derivative thereof) can be produced by transgenic plant transformed with the universal vector of the invention, with suitable adaptations as may be required for a particular case.

occurs only in plants and microorganisms. Unfortunately, because the reaction to form EPSP occurs in all plants, glyphosate does not have selectivity between weeds and desirable plants such as crops and ornamentals.

Two approaches have been used to attempt to develop a glyphosate resistant plant by genetic engineering. One approach is based upon overproduction of wild type EPSP synthase, so that after competitive inhibition of EPSP synthase by glyphosate, the residual EPSP synthase confers glyphosate tolerance. The second approach is based upon the expression of a mutant gene (*aroA*) encoding glyphosate resistant EPSP synthase.

In all of the aforementioned examples, without exception, herbicide resistant genes have been introduced into the nuclear genome.

**The Need for Chloroplast Transformation.** A serious need exists, to develop a herbicide resistant plant, particularly a plant resistant to the most widely used herbicides, in which the protein conferring herbicide resistance is produced in the chloroplast, and in which the gene conferring herbicide resistance cannot escape by pollen to the environment.

The universal vector of the invention responds to this need by transformation of any target plant to provide tolerance to any selected herbicide like to glyphosate. Important commercial crops like wheat, rice, corn (maize), soybean can be made resistant to a selected herbicide by means of the universal vector.

The invention provides a transgenic herbicide resistant plant in which a foreign transgene conferring resistance to one or more herbicide is integrated into the chloroplast genome by means of the universal vector. The transgenic plant may be a mature plant, an immature plant, such as a seedling, an embryo, a callus, a cultured tissue, or cell suspension, or a portion of a plant such as a cutting or a callus. Herbicides which are suitable for the invention and for which genes conferring resistance may be stably integrated

naphthylacetic acid; 3,6-dichloropicolonic acid, picloram, fluoroxypyr, quinclorac, MCPA and 2,4-D.

An additional class are the mitotic herbicides, termed dinitroaniline herbicides, like trifluralin, oryzalin and pendimethalin.

Another chemical class of herbicides to which the invention applies are those acting in the biosynthesis of amino acids, such as tertiary the amino methyl phosphonic acids chlorsulfuron, glufosine and glyphosate.

Another class of herbicides are the acetolactate synthase- inhibiting herbicides (ALS), like the sulfonylureas, imidazolinones, triazolopyrimidines and pyrimidinyl thiobenzoates, such as chlorsulfuron, imazaphyr, flumetsulam (and others listed in chapter 4, Table I of Herbicide Resistance in Plants, 1994, cited below).

Examples of sulfonylurea herbicides are sulfometuron methyl (the active ingredient of Oust™) and chlorsulfuron (the active ingredient of Glean™). Imazapyr, one of the imidazolinones, is the active ingredient of American Cyanamid's herbicide Arsenal™ and imazamethabenz is a mixture of two imidazolinones (Merk Index, 11th Ed. 4825). Mutated forms of ALS located in the structural genes of ALS, *ilvG* and ILV2 may be used to confer herbicide resistance using the universal vector.

In spite of the chemical differences between the imidazolinones and sulfonylureas, these substances inhibit the same enzyme, ALS. It appears that quinones and an imidazolinone herbicide compete with a sulfonylurea herbicide for a common site on ALS. Accordingly, in accordance with the invention, plants can be transformed which will show a resistance to both groups of these and other herbicides.

Another group of chemicals controllable by the invention using the universal vector which has herbicidal activity is typified by L-phosphinothricin, which is a component of the tripeptide "bialaphos". This tripeptide is marketed under the trade name "Herbiace™" by Meiji Seika, Japan, and as "Basta™" by Hoechst AG, Germany. L-

herbicides. The teaching of this invention is readily applicable thereto.

The invention includes a method for producing a herbicide resistant plant which comprises transforming the chloroplast of the plant by introducing one or more foreign transgenes which code for a protein conferring herbicide resistance to the genome of the chloroplast of the plant. Preferably, the transgene codes for a mutant form of the enzyme which has decreased affinity for a given herbicide than does the naturally occurring enzyme.

The following Table lists a variety of type of resistance determinants, (chemicals or "molecules") which inhibit or which confer resistance, and typical herbicides related thereto.

Table I

| RESISTANCE DETERMINANT | HERBICIDES |
|---|---|
| glutathione S-transferase | s-triazine<br>simazine<br>chloracetamide<br>metalachlor |
| Auxin analogs | 2, 4-D<br>MCPA<br>mecopop<br>chloramben |
| EPSP synthase | glyphosate |
| $Q_b$ (*psbA*)- PS II Type | atrazine<br>terbutyne<br>dichloropheny-<br>dimethylurea<br>metribuzine<br>lenacil<br>phenmedipham<br>loxynil<br>dinoseb |
| Acetohydroxyacid sythase (ALS) | sulfonylureas<br>chlorosulfuron<br>imazapyr<br>sulfometuron<br>methyl<br>imidazolinones |
| Glutamine synthase | phosphinothricin |

within this fragment, containing a universal border sequence comprising *trnI* and *trnA* genes (Figure 5A), including the spacer region between the genes, was subcloned into the pUC19 plasmid at the *Pvu*II site (Figure 5B). The resultant plasmid was designated pSBL-Ct Bor (Figure 5C).

The vector pSBL-RD-EPSPS (Figure 2B) contains a mutant EPSP synthase gene that codes for the enzyme EPSP synthase. Glyphosate, the active ingredient in Mosanto's ROUND UP™, binds to the protein EPSP synthase and blocks the synthesis of essential amino acids, resulting in death of a plant. The EPSP synthase coded for by the mutant gene does not bind glyphosate, and therefore confers herbicide resistance to crop plants.

Other genes, such as those that confer resistance to adverse environmental factors such as salt/drought tolerance (osmotolerance genes such as betaine aldehyde dehydrogenase, (BADH), for the overproduction of glycine betaine) or thermotolerance (genes coding for heat shock proteins) or cold shock tolerance proteins, or to pathogen resistance, such as antimicrobial (lytic peptides, chitinase) or antiviral (coat proteins) can be inserted singly or in non-conflicting combinations into the universal chloroplast vector, or into different cassettes of the same universal chloroplast vector to transform the target plant into one with the desired trait.

**Construction of a Universal Chloroplast Integration Vector Containing a Synthetic Spacer 2 Region**

A universal chloroplast vector containing only the spacer 2 region of the tobacco chloroplast genome was constructed by first subcloning a synthetic oligonucleotide comprising the spacer 2 region into the bacterial plasmid pUC19. The positive and negative strands of the 64 base pair spacer sequence were synthesized, the sequence of the positive strand was as follows:

5'-GCTGCGCCAGGGAAAAGAATAGAAGAAGCATCTGACTACTTCATGCATGCTCCACTTGGCTCGG-3'

(100x25 mm) petri plates (about 10 pieces per plate). Selected from the shoots that died, the regenerated spectinomycin resistant shoots were chopped into small pieces (about 2mm$^2$) and subcloned into fresh deep petri plates (about 5 pieces per plate) containing the same lethal selection medium. Resistant shoots from the second culture cycle were transferred to rooting medium (MSO medium supplemented with IBA, 1 μg/liter and an appropriate antibiotic like 500 μg/ml of spectinomycin dihydrochloride,). Rooted plants were transferred to soil and grown at 26°C under continuous lighting conditions for further analysis.

After transfer to the lethal selection medium, the explants gradually became pale and in about 3-8 weeks, green calli and shoots developed from the bombarded side of the 0leaf. Resistant shoots from each callus were considered as a clone.

PCR screening for chloroplast transformants after the first culture cycle showed that 12 out of 20 resistant clones integrate the foreign genes like the *aadA* gene linked to the EG121 gene into the chloroplast genome. These 12 clones were advanced to further steps of regeneration. The entire process of regeneration, starting from bombardment until transfer to soil, takes about 3-5 months.

Figure 9 shows transformed and untransformed tobacco plastids growing in the presence of spectinomycin indicating non-lethal selection on the medium (500μg/ml).

## Example 3

**Corn Chloroplast Transformation.** Surface sterilization and germination of corn seeds. Corn seeds are surface sterilized in a solution containing 20% (v/v) commercial bleach and 0.5% SDS for 15 min under continuous shaking, then serially rinsed in sterile double-distilled water (sddw) four to five times. Liquid MS-based germination medium (modified CSG) containing MS salts (4.3 g/l), sucrose (30 g/l), DM-vitamins (1.0 mg/l thiamine-HCl, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCl and 100 mg/l myo-inositol) and BA (2.0 mg/l) at pH 5.8 is

material to achieve faster growth. Regenerated plantlets are transplanted to potting media, acclimatized then grown to maturity in the greenhouse.

Figure 11 shows corn plastid transformation. Transformed corn plants grow normally (middle shoot) while untransformed plants die on the lethal medium, confirming lethal selection by the antibiotic spectinomycin (1000$\mu$g/ml).

## Example 4

**Rice Chloroplast Transformation.** Surface sterilization of rice seeds and preculture. Dehusked seeds from any genotype (indica or japonica types) are surface sterilized first in 70% ethanol for 10 min under continuous shaking then rinsed with ddw about five times. Seeds are then soaked in a 0.2% Benlate (w/v) solution for 20 min, rinsed with sddw five times, then in 50% bleach for 20 min with the sddw rinses repeated. Seeds are pre-cultured in medium RG1 [MS salts, sucrose and DM-vitamins as above, BA (2.0 mg/l) at pH 5.8]. As with corn, liquid RG1 is dispensed to Magenta™ boxes containing cheesecloth prior to autoclaving. Seeds are placed in RG1 (100 seeds of any genotype per box) and pre-cultured overnight (16 h or continuous light; 23C) prior to bombardment the following day.

**Bombardment of embryos on intact rice seeds.** Pre-cultured seeds are tightly packed vertically, embryo end up (Figure 12A), in the central 2.5 cm area of a petri dish (25 per dish) containing medium RG1.1 (RG1 plus 8.0 g/l phytagar) and bombarded with DNA-coated microprojectiles .

**DNA precipitation.** The procedure is as described for corn with the following modifications. Ten $\mu$l DNA (1.0 $\mu$g/$\mu$l) and 20 $\mu$l isopropanol (2X vol of DNA), 60 $\mu$l 2.5 M $CaCl_2$ and 15 $\mu$l 0.1M spermidine are used. Each shot delivers 2.0 $\mu$g DNA and 720 $\mu$g tungsten.

regeneration.) Multiple shoots were induced on nodal sections excised from three day-old seedlings of 21 genotypes (none related to A188 or B73) which included hybrid (16 grain, one sweet) and inbred (four) genotypes. After eight weeks in culture, 16 - 32 shoots (avg. 24) were generated per explant. Shoots were rooted and regenerants did not display aberrant phenotypes in greenhouse analyses (limited study of two plants per genotype). DNA could also be delivered into nodal section explants of all genotypes (Figure 10C; transient β-glucuronidase expression). For plastid transformation, nodal section explants were bombarded with pSBL-ctV2, then placed on a multiple shoot induction medium containing spectinomycin and streptomycin. Arising shoots could be excised and re-placed on shoot induction medium for subsequent rounds of selection.

As described above unique rice targets dehusked intact mature seeds, embryo end up, not used in previously reported transformation protocols were coupled with a multiple shoot induction protocol (Figure 12) for mature embryos (excised two days post-bombardment). Multiple shoots were induced on all eight genotypes tested (Litton, Priscilla - two newly released Mississippi cultivars, plus six breeding lines). The noted response should be similar in numerous other cultivars since the initial explant is a mature embryo. Regenerants (non-transformed) are being maintained for collection of F1 seed. After plastid transformation, shoot multiplication occurred in the presence of spectinomycin/streptomycin and, as with corn, shoots could undergo numerous rounds of selection due to shoot proliferation (unknown if axillary or adventitious in origin) from the base of excised shoots. Rooting was also accomplished in selective media.

Figures 13A-B shows PCR analysis of DNA isolated from first generation leaves of rice transformants. PCR analysis was done with DNA isolated from the first generation leaves. The PCR products were not abundant as observed in tobacco chloroplast transgenic plants (Figure 13A, lane 11, 13B, lane 12). This may be because of two reasons. The

side of plate) while untransformed plants die in the lethal medium (500 μg/ml).

## Example 6

**Soybean Chloroplast Transformation.** Transgenic soybeans having transformed chloroplast genomes were obtained using the universal vector pSBL-CG-CtV2 (Figure 7A). Bombardment conditions were as for tobacco chloroplast transformation. Soybean chloroplast transformation has never been previously reported.

Figure 15 shows soybean plastic transformation. Two transformed plants show shoots, the other plant die on the lethal medium, confirming lethal selection by the antibiotic spectinomycin (500 μg/ml).

## Example 7

**Sweet Potato Chloroplast Transformation.** Transgenic sweet potato plants having transformed chloroplast genomes were obtained using the universal vector pSBL-CG-CtV2 (Figure 7A). Sweet potato tissue were grown in culture in accordance with the protocol described by Zhang et al.,1996. Bombardment conditions were the same as for tobacco chloroplast transformation as described above, except that calli and primary embryos were bombarded and, after bombardment, were transferred to plates containing 100mg/ml spectinomycin. Sweet potato chloroplast transformation has never been previously reported.

Figure 16 shows sweet potato embryos transformation on the lethal antibiotic spectinomycin selection medium (500 μg/ml). Note bleached calli (right) and green embryos (left).

## Example 8

**Grape Chloroplast Transformation.** Transgenic grape plants having transformed chloroplast genomes are obtained using the same universal vector pSBL-CG-CtV2. Grape tissue are grown in culture according to the protocol of Hebert et al., 1993. All

| | |
|---|---|
| Pasture grass | Embryogenic calli |
| Peach | Embryo derived calli |
| Peanut | Meristems |
| Poplar | Embryogenic |
| Rice | Zygotic embryos |

The transformation of plants by the use of the gene gun is described in Daniell, 1997. Each crop that was reported to be nuclear transformable via microprojectile bombardment in that Table can have its chloroplast genome transformed using the universal vector as described herein.

## Example 10

### Expression of Non-Plant Products

The examples that follow, illustrate the expression of biodegradable protein-based biopolymers (PEPs) and analysis of transformants.

**Vector pSBL-CG-EG121.** The vector pSBL-CG-EG121 (Figure 3A) contains the gene $(GVGVP)_{121mer}$ (designated EG121) which codes for a biodegradable protein-based biopolymer (PBP) that has many medical and non-medical applications.

**Construction of Chloroplast Expression Vectors.** Standard protocols for vector construction were as outlined by Sambrook et al., 1989. Chloroplast integration and expression vectors pSBL-CtV2 (Figure 7A) and pZS197 were digested, respectively, with *Xba*I (an unique site between the *aad*A gene and the *psbA* 3' region) and *Spe*I (a unique site at 120 bp downstream of the *aad*A geen in the *psbA* 3' regulatory region), Klenow filled and dephosphorylated. The polymer gene EG121 along with the Shine-Dalgarno sequence (GAAGGAG) from the pET11d vector was excised as a *Xba*I-*Bam*HI fragment from the plasmid pET11d-EG121. Sticky ends of the insert fragment were Klenow filled and ligated with vectors pSBL-CtV2 or pZS197 yielding chloroplast expression vectors pSBL-CG-EG121 (Figure 3A) and

Total DNA was digested with *Eco*RI and HindIII in case of the universal vector (UV) transformants or *Eco*RI and *Eco*RV in case of the tobacco vector (TV) transformants. Presence of an *Eco*RI site at the 3' end of the polymer gene allowed excision of predicted size fragments in the chloroplast transformants only. To confirm foreign gene integration and homoplasmy, individual blots were probed with corresponding border sequences. In the case of the TV transformants after the second or third round of selection, the border sequence hybridized with 4.6 and 1.6 kbp fragments (Figure 19A, lanes 2,3 and 4) and with a 3.1 kbp native fragment in the wild type (Figure 19A, lane 1). On the other hand, in the case of the UV transformants, after the first round of selection, the border sequence hybridized with 4.0 kbp and 1.2 kbp fragments (Figure 20A, lanes 1 and 2) while it hybridized with a native 2.1 kbp fragment in the control (Figure 20A, lane 3). Moreover, TV transformants also showed the native fragment of 3.1 kbp (Figure 19A, lanes 2 and 3) similar to the wild type plant indicating heteroplasmic condition of the transformed chloroplast genomes, even though they have been under several rounds of selection. However, both UV transformants showed homoplasmic condition, even after the first round of selection (Figure 20A, lanes 1,2).

Presence of heteroplasmy even after second selection was reported earlier and it was suggested that selection should be done until attainment of homoplasmy (Svab and Maliga, 1993). This is consistent with the observation that a high degree of heteroplasmy exists after a second selection cycle in the TV transformants (Figure 19A, lanes 2 and 3). However, no heteroplasmic condition was observed in case of the UV transformants which may be because of the copy correction mechanism between the two IR regions and/or the presence of chloroplast origin of replication (*ori*) within the border sequence, which should increase the copy number of the introduced plasmid before integration.

chloroplasts of transgenic plants. The tobacco vector (TV) integrated plastid transformants showed lower levels of polymer transcript (lanes 1-4) compared to the universal vector integrated transformants (lanes 5,6) because the polymer gene exists as two copies per transformed plastid genome in universal vector transformants as against a single copy in the TV transformants and the heteroplasmic conditions observed in TV transformants.

**Western Blot Analysis**. Polymer protein was purified from leaves from wild type tobacco, chloroplast transformants and nuclear transgenic plants following the method recently described by Zhang et al., 1995. Purified polymer was analyzed by SDS-PAGE according to Laemmli, 1970 using a 12% resolving gel and a 5% stacking gel and run for 5 h at a constant current of 30 mAmps. Polymer polypeptides of about 60 kDa were visualized by negative staining with 0.3 M $CuCl_2$,. Gels were destained in 0.25 M sodium EDTA and 0.25 M Tris-Cl, pH 9.0 with three changes of buffer at 10 min intervals. Western immunoblotting and staining (Figure 22) was carried out as described by Zhang et al., 1996 using a monoclonal antiserum raised against the polymer AVGVP which cross-reacts well with polymer GVGVP and the "Immuno-Blot Assay Kit" (Bio-Rad). The polymer polypeptides running at about 60 kDa are seen in the plastid transformants of IR integrated plants. Polymer expression from a highly expressing F2 generation nuclear transgenic plant (highest expressing plant among 35 transgenic plants examined) is seen in lane 5 (Figure 22), while no polymer was expressed in the untransformed control as seen in lane 4 (Figure 22). Eleven to fifty fold higher level of polymer transcripts is shown in the chloroplast transformants (Figure 21). In the case of chloroplast native occurring proteins like valine and proline whose biosynthetic pathways are compartmentalized in chloroplasts, higher levels of protein can be expected to be produced.

**Integration of the Gene.** Fully expanded green leaves of *Nicotiana tabaccum* var. Petit Havana were bombarded with the tobacco and the universal chloroplast vectors. Two days after bombardment, leaf explants were transferred to selection lethal medium containing spectinomycin (500 μg/ml). Transgenic plants were obtained within 3-5 months after bombardment. Typically, out of 16 bombarded leaves, 10 independently transformed shoots were identified.

PCR analysis was performed with DNA isolated from the first or second generation shoots and also from the mature transgenic plants. Primers were used to confirm integration of the *aadA* gene into the plant genome from the tobacco as well as universal vectors. Lack of a product would indicate spontaneous mutants, capable of growing on spectinomycin without the *aadA* gene. The expected PCR product (887bp) was obtained from six lines (Figures 24A-B, lanes 1-6) transformed with the tobacco vector. A PCR product of 1.57Kb was detected in four lines (Figures 24A-B, lanes 1-4) transformed with the universal vector. Under the selection conditions used, four mutants were detected out of ten lines transformed with the tobacco vector. On the other hand, all the transgenic lines transformed with the universal vector showed integration of the *aadA* gene.

**PCR Chloroplast Integration.** Primers were also designed to specifically identify integration into the plastid genome. The strategy here was to land one primer on the native chloroplast genome, adjacent to the point of integration of the vector, while landing the other on the *aadA* gene. A primer was designed to land immediately outside the *rbcL* gene in the tobacco vector (2.08Kb PCR product). For the universal vector, the primer on the native chloroplast genome landed in the 16s rRNA gene (1.60Kb PCR product). The expected products were observed for the transgenic lines obtained using the tobacco vector (Figures 24A-B, lanes 2-7) as well as the universal vector (Figures 24A-B, lanes 1-4). Unbombarded plants

chloroplast genome. Tobacco chloroplasts contain 5000-10,000 copies of their genome per cell. (McBride et al, 1995) If only a fraction of the genomes are actually transformed, the copy number, by default, must be less than 10,000. By establishing that in the transgenics the EPSPS transformed genome is the only one present, one could establish that the copy number is 5000-10,000 per cell. This was shown by digesting the total DNA with *Eco*RI and probing, with the flanking sequences that enable homologous recombination into the chloroplast genome. The probe comprised a 2.9 Kb fragment of the *rbcL-orf* 512 sequences. A chloroplast genome transformed with the EPSPS gene, incorporates an *Eco*RI site between the *rbcL-orf* 512 region of the chloroplast genome, thereby generating an extra fragment when digested with this enzyme (Figure 25C). Southern hybridization analysis revealed a 4.43 Kb fragment in Figure 25B, lane 1 for the untransformed control. In lanes 2,3 and 4, two fragments (4.35 Kb and 3 Kb) were generated due to the incorporation of the EPSPS gene cassette between the *rbcL* and *orf*512 regions (Figure 25C provides a schematic diagram with the dotted lines in gray signifying the point of integration of the foreign DNA). The 4.43 Kb fragment present in the control is absent in the transgenics. This proves that only the transgenic chloroplast genome is present in the cell and there is no native, untransformed, chloroplast genome, without the EPSPS gene present. This establishes the homoplasmic nature of the transformants, simultaneously providing an estimate of 5000-10,000 copies of the foreign EPSPS gene per cell. This would then explain the high levels of tolerance of glyphosate that was observed in the transgenic tobacco plants (Figure 20A).

**Progeny.** Seeds collected from self-pollinated transgenic plants were germinated in the presence of spectinomycin (500 µg/ml). All seeds germinated, remained green and grew normally (Figure 26B). Uniform spectinomycin resistance

## Example 12

**Tolerance of Corn to Glyphosate.** A universal chloroplast vector using corn chloroplast DNA is constructed as follows. First, vector pSBL-Ct-bor (Figure 5C) is constructed as follows: Corn chloroplast DNA subclone containing one of the inverted repeat regions is constructed with bacterial plasmid pUC19. Second, a smaller subclone containing only the rRNA operon is constructed from the first subclone and the fragment present in the second subclone containing the *trnA* and *trnI* genes and spacer regions representing the universal border are subcloned into a pUC19 plasmid at the *Pvu*II site. The resultant plasmid is designated pSBL-Ct-bor. Within plasmid pSBL-Ct-bor, a selectable maker gene cassette containing a chloroplast 16S rRNA promoter, the *aadA* gene (encoding aminoglycoside 3'adenyl transferase conferring resistance for streptomycin/spectinomycin) and a 3' untranslated region of the chloroplast *psbA* gene is inserted to construct vector pSBL-CORN. The selectable maker gene cassette is inserted between the *trnI* and *trnA* genes in the spacer region, in the direction of the 16S rDNA transcription.

The vector pSBL-CORN-aroA, which contains a mutant *aroA* gene from *Salmonella typhimurium* (Stalker et al. 1985; Comai et al. 1983)that encodes the enzyme EPSPS synthase, is constructed by inserting the mutant aroA gene into the pSBL-CORN vector. Transgenic corn plants expressing the mutant aroA gene are resistant to glyphosate treatment like "Roundup™" whereas the untransformed control plants are not.

## Example 13

**Chloroplast Transformation for Tolerance to Imidizolinones or Sulfonylureas**. Plasmid pSBL-CORN is modified by insertion of a DNA fragment containing a mutated form of the acetolactate synthase gene of *Saccharomyces cerevisiae* (Falco and Dumas. 1985; Yadav et al. 1986) to generate plasmid pSBL-CORN-ASL1. This gene encodes an acetolactate synthase that is not

utilized to transform plants. Daniell (1997). Incorporation of the mutant *psbA* genes into the chloroplast genome and selection of the appropriate transformants are carried out as previously described. Transformed plants producing the mutated *psbA* protein containing the serine to glycine substitution are resistant to Atrazine™ whereas control plants are not.

The mutant *psbA* gene containing a valine to isoleucine mutation at residue 219 is isolated from genomic DNA of *Chlamydomonas* using the appropriate restriction endonucleases. A universal vector is constructed as described above. Transgenic plants like corn expressing *psbA* containing the valine to isoleucine mutation at residue 219 are expected to be resistant to DCMU sprays.

## Example 15

**Tolerance to Auxin Analogs. 2,4-D.** The universal chloroplast expression vector *psbL*-ctV2 can be cleaved with *XbaI* and ligated with a DNA fragment containing a gene encoding monooxygenase. The resulting construct can be transformed into chloroplasts to generate transgenic plants that contain multiple copies of the monooxygenase gene. The resulting plants expressing high levels of monooxygenase and are expected to be tolerant to 2,4-D.

## Example 16

**Chloroplast Transformation for Insect Resistance.** Tobacco plants can be transformed with universal vector pSBL-CtVHBt (Figure 8A) which contain the *cry*IIA gene and expresses the CryIIA protoxin, thereby conferring resistance to insects pests like of the family Pyralidoe, such as the tobacco hornworm. Even insects which have developed a resistance or are less susceptible to Bt toxin are killed by the Bt toxin expressed by the gene in the chloroplast vector described herein.

Other controllable insects are described earlier in the description of the invention.

As will be apparent to those skilled in the art, in light of the foregoing description, many modifications, alterations, and substitutions are possible in the practice of the invention without departing from the spirit or scope thereof. It is intended that such modifications, alterations, and substitutions be included in the scope of the claims.

All references cited in this text are expressly incorporated herein by reference.

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## CLAIMS

What is claimed is:

1. A universal integration and expression vector competent for stably transforming the chloroplast genome of different plant species which comprises an expression cassette which comprises, operably joined, a heterologous DNA sequence coding for a molecule of interest and control sequences positioned upstream from the 5' and downstream from the 3' ends of the coding sequence to provide expression of the coding sequence in the chloroplast genome of a target plant, and flanking each side of the expression cassette, flanking DNA sequences which are homologous to a spacer sequence of the target chloroplast genome, which sequence is conserved in the chloroplast genome of different plant species, whereby stable integration of the heterologous coding sequence into the chloroplast genome of the target plant is facilitated through homologous recombination of the flanking sequences with the homologous sequences in the target chloroplast genome.

2. A universal integration and expression vector competent for stably transforming the chloroplast genome of different plant species which comprises an expression cassette which comprises, operably joined, a heterologous DNA sequence coding for a peptide of interest and control sequences positioned upstream from the 5' and downstream from the 3' ends of the coding sequence to provide expression of the coding sequence in the chloroplast genome of a target plant, and flanking each side of the expression cassette, flanking DNA sequences which are homologous to a spacer sequence of the target chloroplast genome, which sequence is conserved in the chloroplast genome of different plant species, whereby stable integration of the heterologous coding sequence into the chloroplast genome of the target plant is facilitated through homologous recombination of the flanking sequences with the homologous sequences in the target chloroplast genome.

3. The vector of claim 2 which comprises a heterologous nucleotide sequence coding for a selectable phenotype.

13. The vector of claim 7 which comprises in the spacer region, a chloroplast origin of replication, whereby homoplasmy with the genome is promoted.

14. The vector of claim 8 which comprises in the spacer region, a chloroplast origin of replication, whereby homoplasmy with the genome is promoted.

15. The vector of claim 9 which comprises in the spacer region, a chloroplast origin of replication, whereby homoplasmy with the genome is promoted.

16. The vector of claim 4 wherein the DNA of the flanking sequences originate from a plant species other than that of the target plant.

17. The vector of claim 5 wherein the DNA of the flanking sequences originate from a plant species other than that of the target plant.

18. The vector of claim 6 wherein the DNA of the flanking sequences originate from a plant species other than that of the target plant.

19. The vector of claim 7 wherein the DNA of the flanking sequences originate from a plant species other than that of the target plant.

20. The vector of claim 8 wherein the DNA of the flanking sequences originate from a plant species other than that of the target plant.

21. The vector of claim 9 wherein the DNA of the flanking sequences originate from a plant species other than that of the target plant.

22. The vector of claim 10 wherein the DNA of the flanking sequences originate from a plant species other than that of the target plant.

23. The vector of claim 11 wherein the DNA of the flanking sequences originate from a plant species other than that of the target plant.

34. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 7, or the progeny thereof.

35. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 8, or the progeny thereof.

36. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 9, or the progeny thereof.

37. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 10, or the progeny thereof.

38. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 11, or the progeny thereof.

39. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 12, or the progeny thereof.

40. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 25, or the progeny thereof.

41. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 26, or the progeny thereof.

42. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 27, or the progeny thereof.

43. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 28, or the progeny thereof.

44. A stably transformed plant which comprises chloroplast stably transformed with the vector of claim 29, or the progeny thereof.

59. The stably transformed plant of claim 35 which is monocotyledonous.

60. The stably transformed plant of claim 36 which is monocotyledonous.

61. The stably transformed plant of claim 34 which is dicotyledonous.

62. The stably transformed plant of claim 35 which is dicotyledonous.

63. The stably transformed plant of claim 36 which is dicotyledonous.

64. The stably transformed plant of claim 37 which is monocotyledonous.

65. The stably transformed plant of claim 38 which is monocotyledonous.

66. The stably transformed plant of claim 39 which is monocotyledonous.

67. The stably transformed plant of claim 37 which is dicotyledonous.

68. The stably transformed plant of claim 38 which is dicotyledonous.

69. The stably transformed plant of claim 39 which is dicotyledonous.

70. The stably transformed plant of claim 40 which is monocotyledonous.

71. The stably transformed plant of claim 41 which is monocotyledonous.

72. The stably transformed plant of claim 42 which is monocotyledonous.

85. A process for stably transforming a target plant species which comprises introducing an integration and expression universal vector into the chloroplast genome of the target plant and allowing the transformed plant to grow, the vector being competent to stably transform the chloroplast of different plant species and comprising an expression cassette which comprises, operably joined, a heterologous DNA sequence coding for a molecule of interest and control sequences positioned upstream from the 5' and downstream from the 3' ends of the coding sequence to provide expression of the coding sequence in the chloroplast of the target plant, a heterologous sequence coding for a selectable phenotype, and flanking each side of the expression cassette, flanking DNA sequences which comprise each one a portion of the intergenic spacer 2 region between the $tRNA^{Ile}$ and the $tRNA^{Ala}$ genes of the chloroplast genome, which are homologous to a spacer sequence of the target chloroplast genome, which sequence is conserved in the chloroplast genome of different plants species, whereby stable integration of the heterologous coding sequence into the chloroplast genome of the target plant is facilitated through homologous recombination of the flanking sequences with the homologous sequences in the target chloroplast genome.

86. A process for stably transforming a target plant species which comprises introducing an integration and expression universal vector into the chloroplast genome of the target plant species and allowing the transformed plant to grow, the vector being competent to stably transform the chloroplast of different plants species and comprising an expression cassette which comprises, operably joined, a heterologous DNA sequence coding for a peptide of interest, and control sequences positioned upstream from the 5' and downstream from the 3' ends of the coding sequence to provide expression of the coding sequence in the chloroplast of the target plant, a heterologous nucleotide sequence coding for a selectable phenotype, and flanking each side of the expression cassette, flanking DNA sequences which comprise each one a portion of the intergenic spacer 2 region between the $tRNA^{Ile}$ and the $tRNA^{Ala}$ genes of the chloroplast genome, which are homologous to a spacer sequence of the target chloroplast genome, which sequence is

98. The process of claim 97 wherein the PBP has repeating pentamer sequences $(GVGVP)_n$ wherein "n" is an integer of 1 to 250, "G" is glycine, "V" is valine and "P" is proline.

99. The process of claim 98 wherein "n" is 121.

100. The process of claim 96 wherein the expressed polypeptide of interest is insulin.

101. The process of claim 100 which comprises isolating the insulin.

102. The process of claim 100 wherein the insulin is in the form of pro-insulin.

103. The process of claim 102 wherein the pro-insulin is fused to a PBP.

104. The process of claim 100 wherein the transformed plant is tobacco.

105. The process of claim 96 wherein the polypeptide of interest is human serum albumin (HSA).

106. The process of claim 105 wherein the transformed plant is tobacco.

107. The vector of claim 4 wherein the peptide of interest is a biologically active molecule.

108. The vector of claim 107 wherein the peptide is a polypeptide which is a synthetic protein-based polymer (PBP).

109. The vector of claim 108 wherein the PBP has repeating pentamer sequences $(GVGVP)_n$ wherein "n" is an integer of 1 to 250, "G" is glycine, "V" is valine and "P" is proline.

110. The vector of claim 109 wherein the polypeptide is $(GVGVP)_n$ wherein the integer "n" is 121.

in the chloroplast genome of the target plant species, a selected phenotype other than tolerance to said herbicide, and flanking each side of the expression cassette, flanking DNA sequences which comprise each one a portion of the intergenic spacer 2 region between the $tRNA^{Ile}$ and the $tRNA^{Ala}$ genes of the chloroplast genome, which are homologous to a spacer sequence of the target chloroplast genome, which sequence is conserved in the chloroplast genome of different plant species, whereby stable integration of the heterologous protein into the chloroplast genome of the target plant was facilitated through homologous recombination of the flanking sequences with the homologous sequences in the target chloroplast genome.

124. The herbicide resistant target plant of claim 123 in which the protein of interest is a mutant form of an enzyme which has decreased affinity for the herbicide than does the naturally occurring enzyme.

125. The herbicide resistant target plant of claim 123 wherein the herbicide is glyphosate.

126. The herbicide resistant target plant of claim 125 wherein the enzyme is EPSP synthase.

127. The herbicide resistant target plant of claim 124 wherein the herbicide is selected from at least one of the following types: PSI, PSII, APP, auxin analog, mitotic, tertiary amino methyl phosphoric acids type and ALS inhibiting types.

128. The herbicide resistant target plant of claim 127 wherein the herbicide is the PSI type selected from paraquat and diquat.

129. The herbicide resistant target plant of claim 127 wherein the herbicide is selected from atrazine, dinoseb, lenacil and metribuzine.

130. The herbicide resistant target plant of claim 127 wherein the herbicide is of the APP type selected from cyclohexanedione, haloxyfop, clethodim and phenoxaprop, and the lower alkyl-substituted compound thereof.

species which comprises an expression cassette which comprises, operably joined, a heterologous DNA sequence coding for a protein of interest which confers resistance to a herbicide and control sequences positioned upstream from the 5' and downstream from the 3' ends of the coding sequence to provide expression of the coding sequence in the chloroplast of the target plant, a heterologous nucleotide sequence coding for a selectable phenotype other than for tolerance to said herbicide, and flanking each side of the expression cassette, flanking sequences which comprise each one a portion of the intergenic spacer 2 region between the $tRNA^{Ile}$ and the $tRNA^{Ala}$ genes of the chloroplast genome, which are homologous to a spacer sequence of the target chloroplast genome, which sequence is conserved in the chloroplast genome of different plant species, whereby stable integration of the heterologous coding sequence into the chloroplast genome of the target plant is facilitated through homologous recombination of the flanking sequences with the homologous sequences in the target chloroplast genome and growing the transformed plant.

141. The process of claim 140 wherein the DNA sequence codes for a mutant form of an enzyme which has decreased affinity for the herbicide than does the naturally occurring enzyme.

142. The process of claim 141 wherein the enzyme is EPSP synthase and the herbicide is glyphosate.

143. The process of claim 142 wherein DNA sequence is the EPSP synthase gene which is a mutant EPSP synthase gene.

144. The process of claim 140 which comprise selecting the viable, transformed target plants from a medium which is lethal to non-transformed plants.

145. The process of claim 144 wherein the viable transformed target plants are homoplasmic plants.

146. The process of claim 144 wherein the viable transformed target plants are heteroplasmic plants.

to a spacer sequence of the target chloroplast genome, which sequence is conserved in the chloroplast genome of different plant species, whereby stable integration of the heterologous coding sequence into the chloroplast genome of the target plant is facilitated through homologous recombination of the flanking sequences with the homologous sequences in the target chloroplast genome, exposing the plants into which the vector has been introduced to a lethal concentration of the herbicide and selecting the plants which do not die from exposure thereto, thereby having selected the transformed plants which express the desired target trait.

153. The process of claim 152 wherein the selected herbicide is selected from at least one of the following types: PSI, PSII, APP, auxin analog, mitotic, tertiary amino methyl phosphoric acids and ALS inhibiting types.

154. A stably transformed insect resistant target plant species or the progeny thereof, which comprises chloroplast genome stably transformed with a universal integration and expression vector competent for stable transformation of the chloroplast of different plants species which comprises an expression cassette which comprises a heterologous DNA sequence which expresses a target protein which confers resistance to a target insect in the chloroplast genome of the target plant species, a selected phenotype other than tolerance to the target insect, and flanking each side of the expression cassette, flanking DNA sequences which comprise each one a portion of the intergenic spacer 2 region between the $tRNA^{Ile}$ and the $tRNA^{Ala}$ genes of the chloroplast genome, which are homologous to a spacer sequence of the target chloroplast genome, which sequence is conserved in the chloroplast genome of different plants species, whereby stable integration of the heterologous protein into the chloroplast genome of the target plant was facilitated through homologous recombination of the flanking sequences with the homologous sequences in the target chloroplast genome, wherein the heterologous protein is the CryIIA protein toxin expressed by the *cry*IIA gene, which protein confers resistance to insects.

164. The isolated intergenic DNA sequence of claim 163 which comprises the trnI and the trnA genes.

165. The isolated DNA sequence of claim 163 which comprises one of the inverted repeats of the chloroplast genome of the plant.

166. The isolated DNA sequence of claim 165 which comprises an origin of replication.

167. The isolated DNA sequence of claim 163 which comprises the rRNA operon in the spacer 2 region.

168. The universal integration and expression vector of claim 4 wherein the spacer 2 region comprises the rRNA operon.

169. The universal integration and expression vector of claim 4 wherein the flanking sequences are synthetic.

170. The herbicide resistant target plant species of claim 85 wherein the DNA sequence which codes for the protein of interest is of prokaryotic origin.

171. The universal integration and expression vector of claim 2 which does not include a transposon.

172. The stably transformed target plant species of claim 41 which does not include a transposon.

173. The process for stably transforming a target plant species of claim 86 wherein the universal vector does not include a transposon.

174. The universal integration and expression vector of claim 4 which comprises a promoter functional in chloroplast.

175. The Universal expression and integration vector of claim 4 which comprises a nucleotide sequence encoding a selectable phenotype which allows for

183. A stably transformed transcription/ translation active chloroplast genome of a target plant, which is competent for stable integration of a heterologous DNA sequence, which comprises an expression cassette which comprises a heterologous molecule of interest encoded by a heterologous DNA sequence and expressed by control sequences in the chloroplast genome of the target plant, and plant DNA flanking each side of the expression cassette which facilitated stable integration of the DNA into the target chloroplast genome by homologous recombination, which DNA is inherited through organelle replication in daughter cells.

184. The stably transformed chloroplast of claim 179 wherein the molecule of interest is a protein-based polymer (PBP).

185. The stably transformed chloroplast of claim 180 wherein the PBP has repeating pentamer sequences $(GVGVP)_n$ wherein n is an integer of 1 to 250, "G" is glycine, "V" is valine and "P" is proline.

186. The stably transformed chloroplast of claim 181 wherein the synthetic coding sequence is the synthetic biopolymer gene EG121 and the expressed PBP is the polymer protein $(GVGVP)_{121}$.

187. The synthetic biopolymer $(GVGPP)_n$ which is expressed from the stably transformed chloroplast of claim 182, wherein "n", "G", "V" and "P" are defined therein.

188. The synthetic biopolymer of claim 183 which is $(GVGVP)_{121}$.

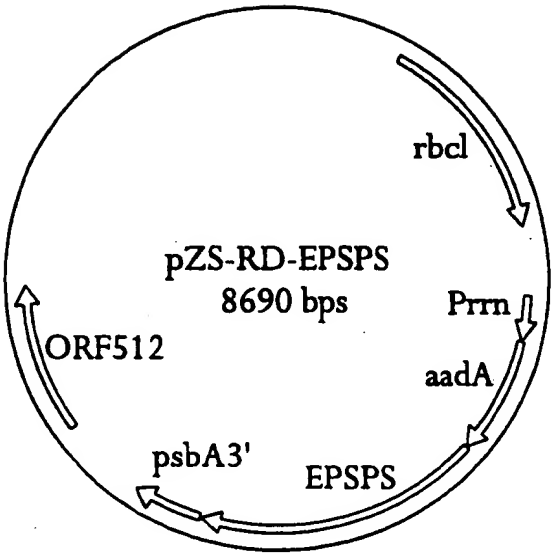



FIG. 2A

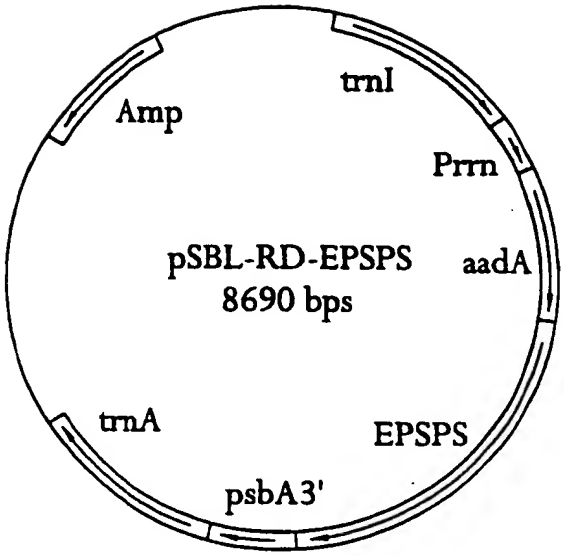



FIG. 2B

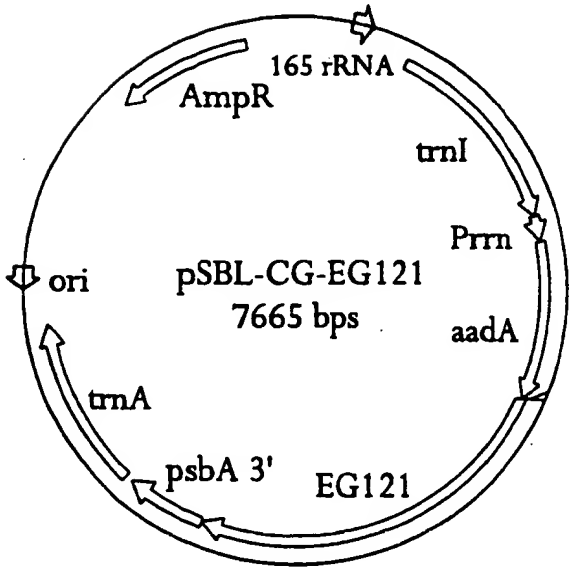



FIG. 3A

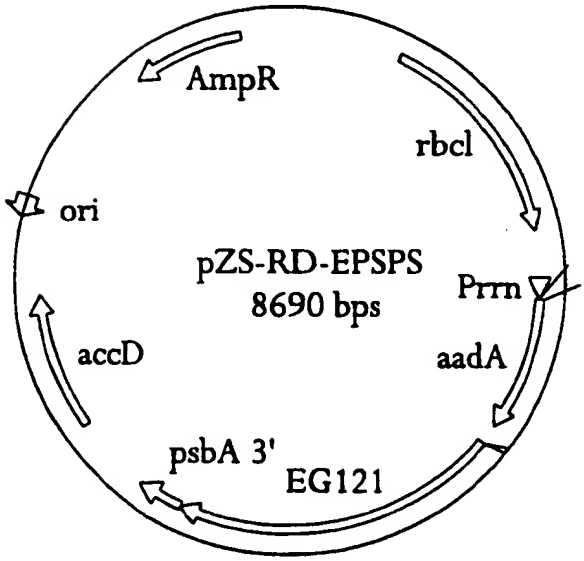



FIG. 3B

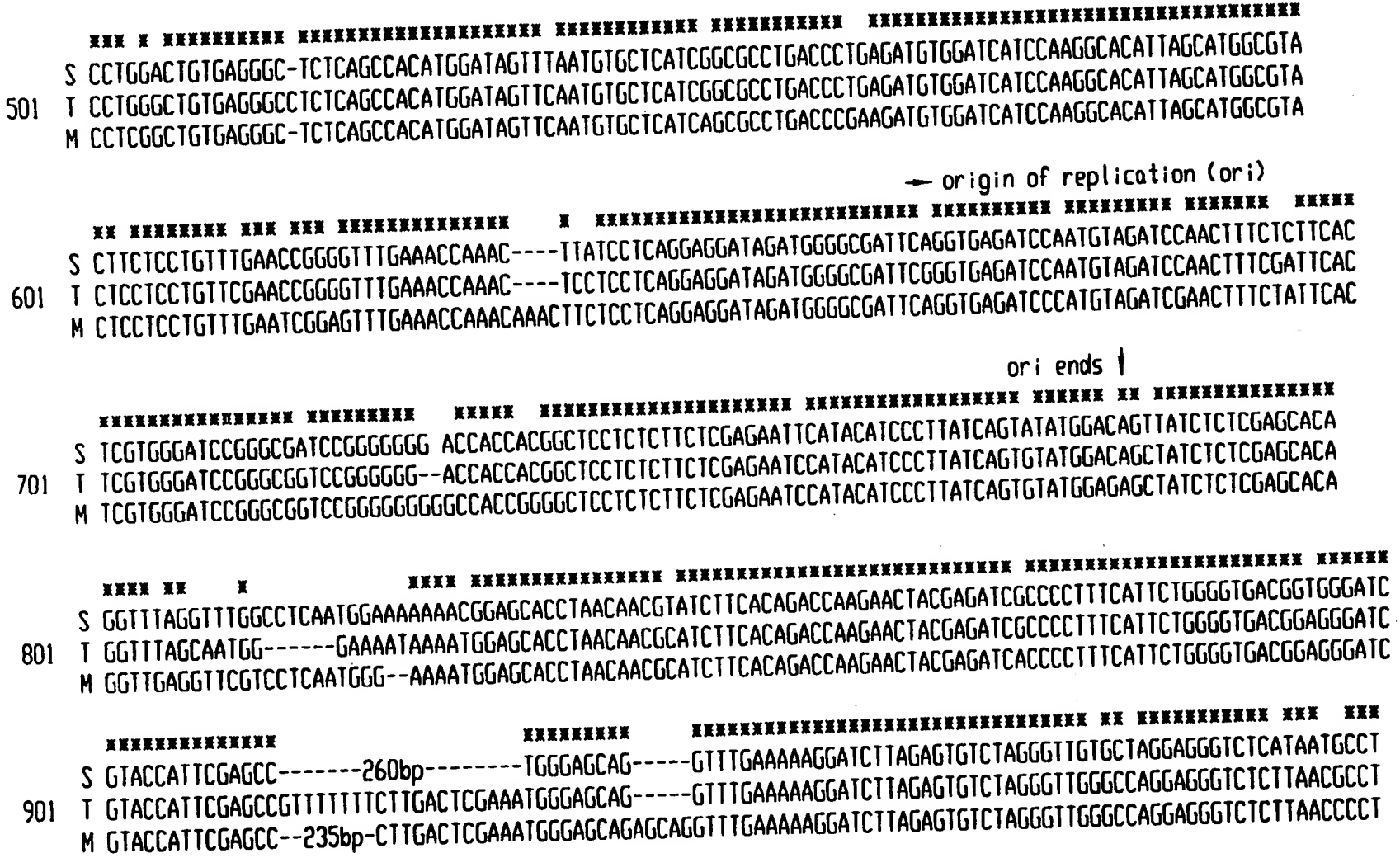



FIG. 4B

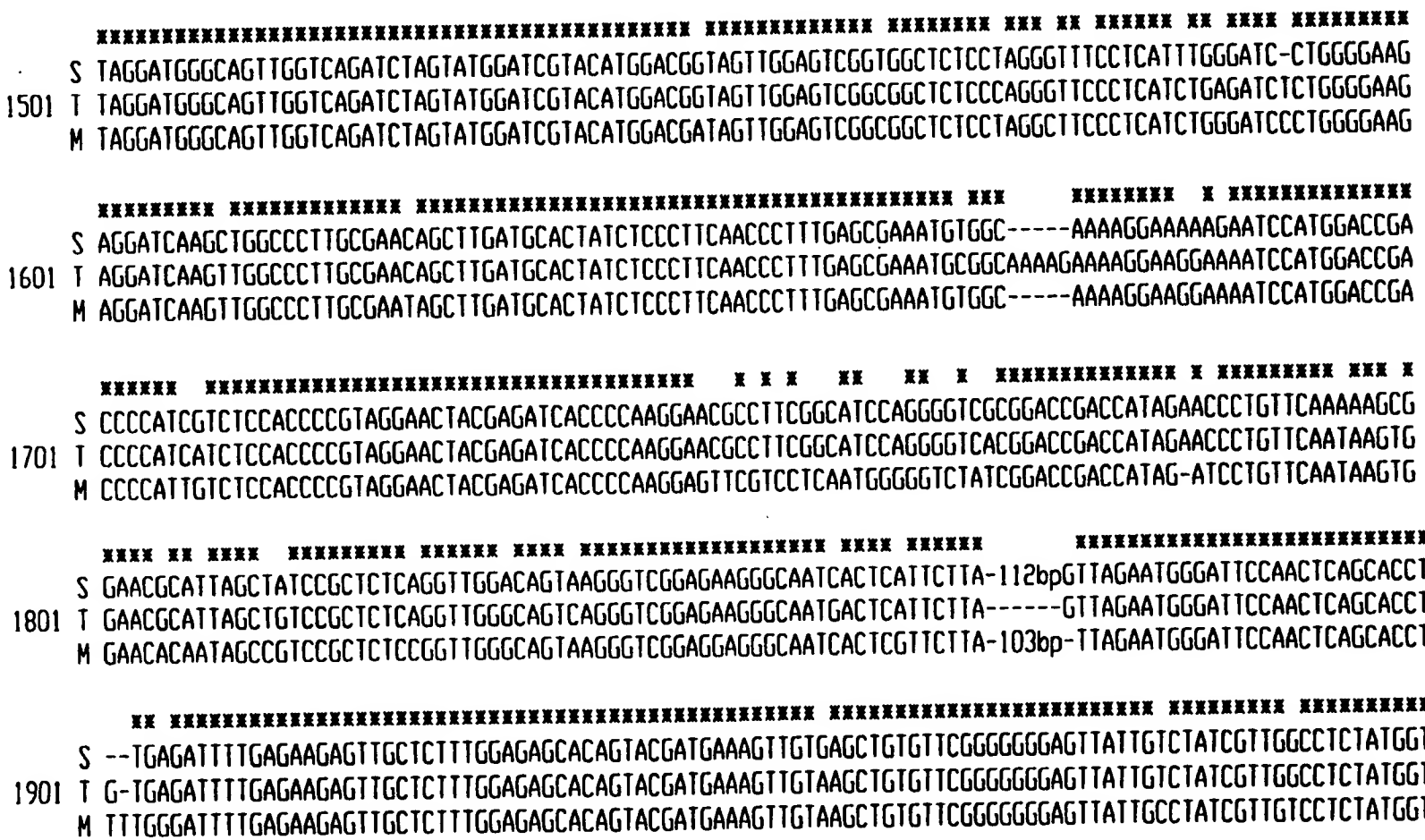
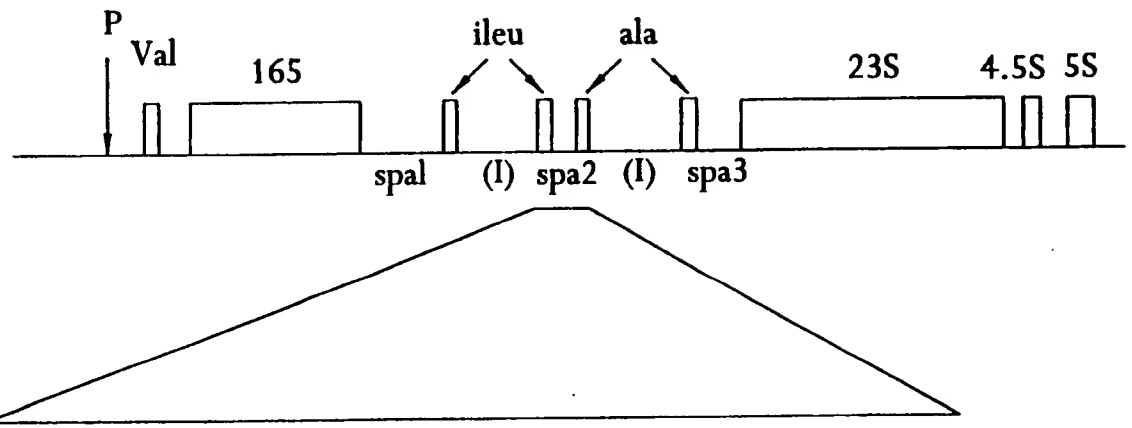

S TAGGATGGGCAGTTGGTCAGATCTAGTATGGATCGTACATGGACGGTAGTTGGAGTCGGTGGCTCTCCTAGGGTTTCCTCATTTGGGATC-CTGGGGAAG
1501 T TAGGATGGGCAGTTGGTCAGATCTAGTATGGATCGTACATGGACGGTAGTTGGAGTCGGCGGCTCTCCCAGGGTTCCCTCATCTGAGATCTCTGGGGAAG
M TAGGATGGGCAGTTGGTCAGATCTAGTATGGATCGTACATGGACGATAGTTGGAGTCGGCGGCTCTCCTAGGCTTCCCTCATCTGGGATCCCTGGGGAAG
S AGGATCAAGCTGGCCCTTGCGAACAGCTTGATGCACTATCTCCCTTCAACCCTTTGAGCGAAATGTGGC-----AAAAGGAAAAAGAATCCATGGACCGA
1601 T AGGATCAAGTTGGCCCTTGCGAACAGCTTGATGCACTATCTCCCTTCAACCCTTTGAGCGAAATGCGGCAAAAGAAAAGGAAGGAAAATCCATGGACCGA
M AGGATCAAGTTGGCCCTTGCGAATAGCTTGATGCACTATCTCCCTTCAACCCTTTGAGCGAAATGTGGC-----AAAAGGAAGGAAAATCCATGGACCGA
S CCCCATCGTCTCCACCCCGTAGGAACTACGAGATCACCCCAAGGAACGCCTTCGGCATCCAGGGGTCGCGGACCGACCATAGAACCCTGTTCAAAAAGCG
1701 T CCCCATCATCTCCACCCCGTAGGAACTACGAGATCACCCCAAGGAACGCCTTCGGCATCCAGGGGTCACGGACCGACCATAGAACCCTGTTCAATAAGTG
M CCCCATTGTCTCCACCCCGTAGGAACTACGAGATCACCCCAAGGAGTTCGTCCTCAATGGGGGTCTATCGGACCGACCATAG-ATCCTGTTCAATAAGTG
S GAACGCATTAGCTATCCGCTCTCAGGTTGGACAGTAAGGGTCGGAGAAGGGCAATCACTCATTCTTA-112bpGTTAGAATGGGATTCCAACTCAGCACCTTT---
1801 T GAACGCATTAGCTGTCCGCTCTCAGGTTGGGCAGTCAGGGTCGGAGAAGGGCAATGACTCATTCTTA------GTTAGAATGGGATTCCAACTCAGCACCTTTTGA
M GAACACAATAGCCGTCCGCTCTCCGGTTGGGCAGTAAGGGTCGGAGGAGGGCAATCACTCGTTCTTA-103bp-TTAGAATGGGATTCCAACTCAGCACCTTTTGT
S --TGAGATTTTGAGAAGAGTTGCTCTTTGGAGAGCACAGTACGATGAAAGTTGTGAGCTGTGTTCGGGGGGGAGTTATTGTCTATCGTTGGCCTCTATGGT
1901 T G-TGAGATTTTGAGAAGAGTTGCTCTTTGGAGAGCACAGTACGATGAAAGTTGTAAGCTGTGTTCGGGGGGGAGTTATTGTCTATCGTTGGCCTCTATGGT
M TTTGGGATTTTGAGAAGAGTTGCTCTTTGGAGAGCACAGTACGATGAAAGTTGTAAGCTGTGTTCGGGGGGGAGTTATTGCCTATCGTTGTCCTCTATGGT

FIG. 4D

GENE SEQUENCE OF rRHA CODING REGIONS IN PLASTID DNA FROM HIGHER PLANTS




SEQUENCE ALIGNMENT OF SPACER-2 (64 bp) REGION FROM SEVERAL CROP SPECIES WITH TOBACCO

```
EPIFAGUS  (90  %)     GCTGCGCTA-GGAAAAAAATATAAAAAGCATCTGATTACTTCATGCATGCT
TOBACCO (+)---      GCTGCGCCAGGGAAAAGAATAGAAGAAGCATCTGACTACTTCATGCATGCTCCA-CTTGGCTCGG
                    **** ** * * **** * ** *      *****  ********** ***** ** ******
HELIANTHUS(96  %)     CGTGCGCCAGGGAAAAGAATAGAAGAAGCGTCTGACTCCTTCATGCATGCTCCA-CTTGGCTCGG
OENOTHERA (96  %)     GCTGCGCAAAGGAAAAGAATAGAAGAAGCATCTGACTCCTTCATGCATGCTCCA-CTTGGCTCGG
ALNUS     (95  %)     GCTGCGCCAAGTAAAAGAATAGAAGAAGCATCTGACTCCTTCATGCATGCTCCA-CTTGGCTCGG
RICE      (95  %)     GCTGCGCCAGGGAAAAGAATAGAAGAAGCATCTGACTCTTTCATGCATACTCCA-CTTGGCTCGG
MAIZE     (94  %)     GCTGCG-CAGGGAAAAGAATAGAAGAAGCATCTGACTCTTTCATGCATACTCCA-CTTGGCTCGG
SOYBEAN   (84  %)     GCTGCGTCAAGGAAAAGAATAGAAAACTGACTTGACTCCTTCATGCATGCTCCA-CTCGGCTCGG
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FIG. 4F

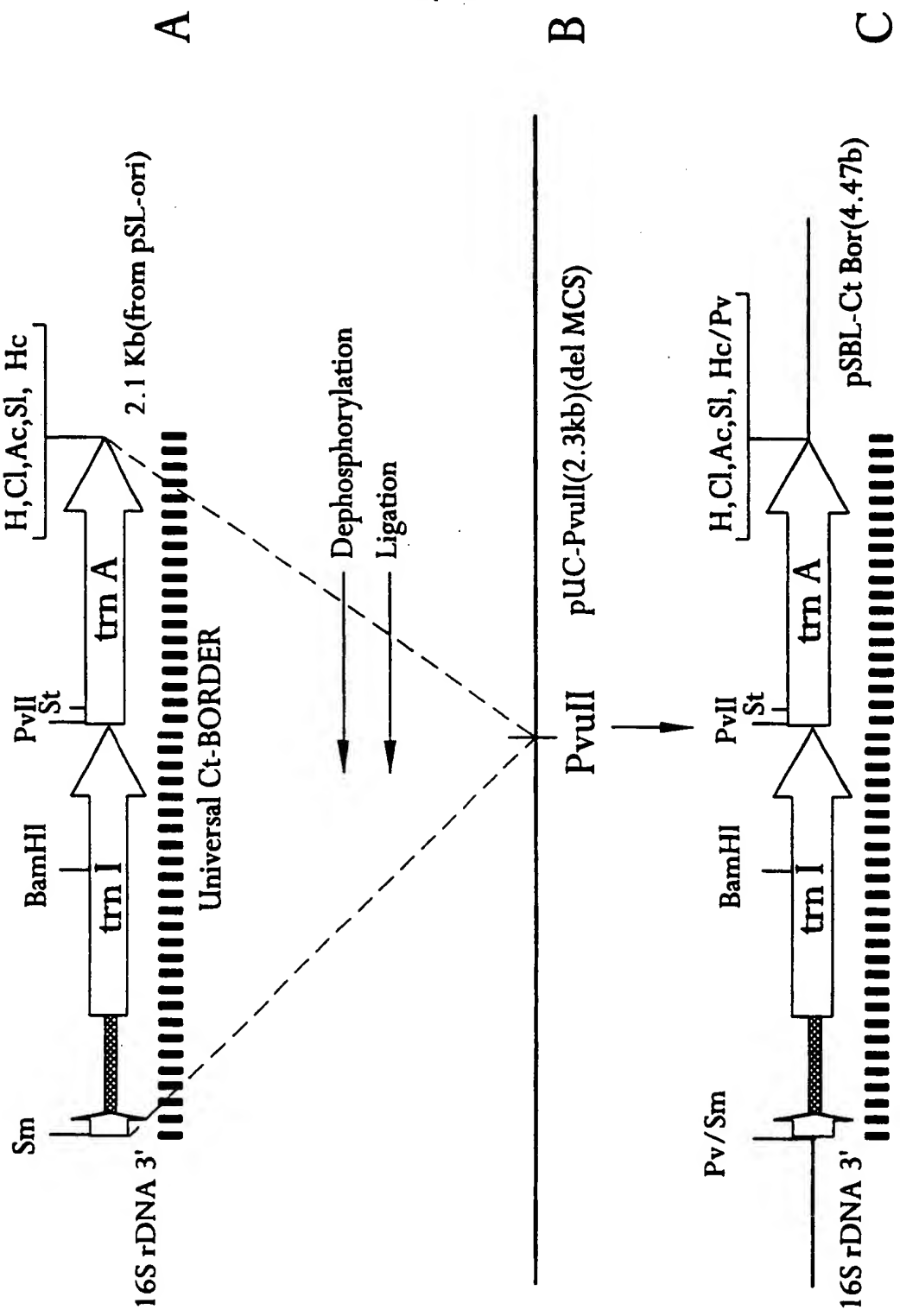


FIG. 5

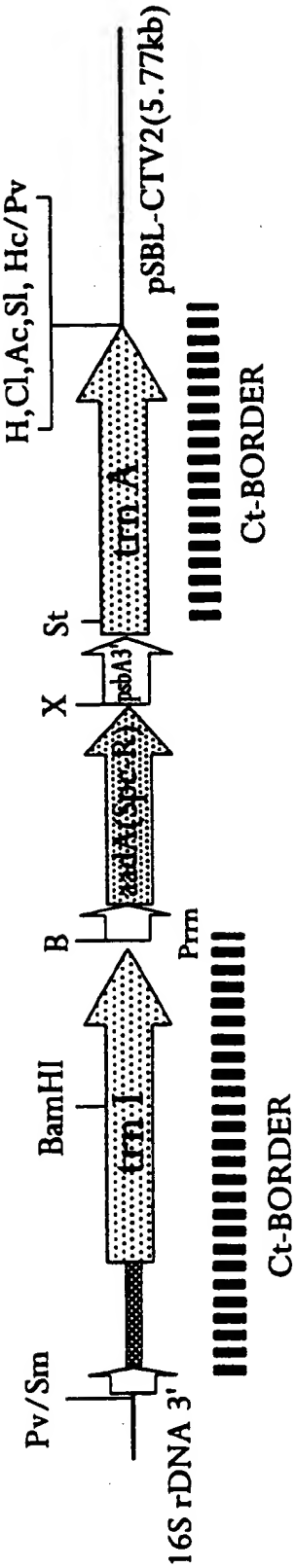


FIG. 7A

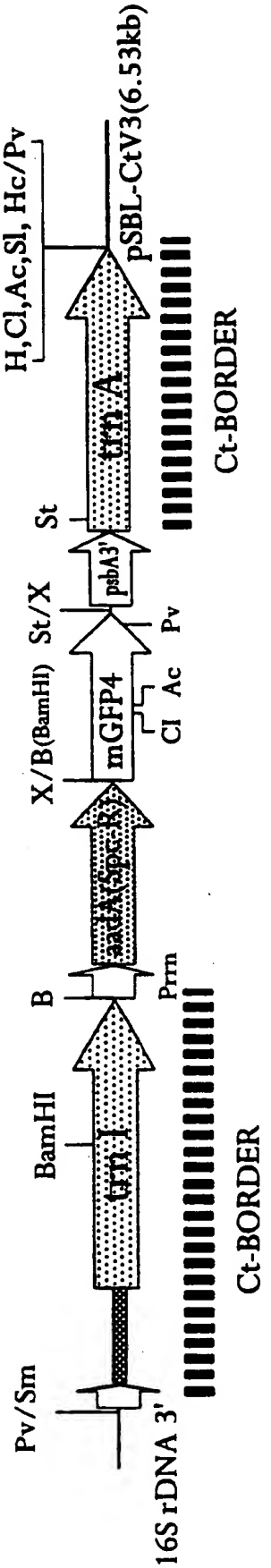


FIG. 7B

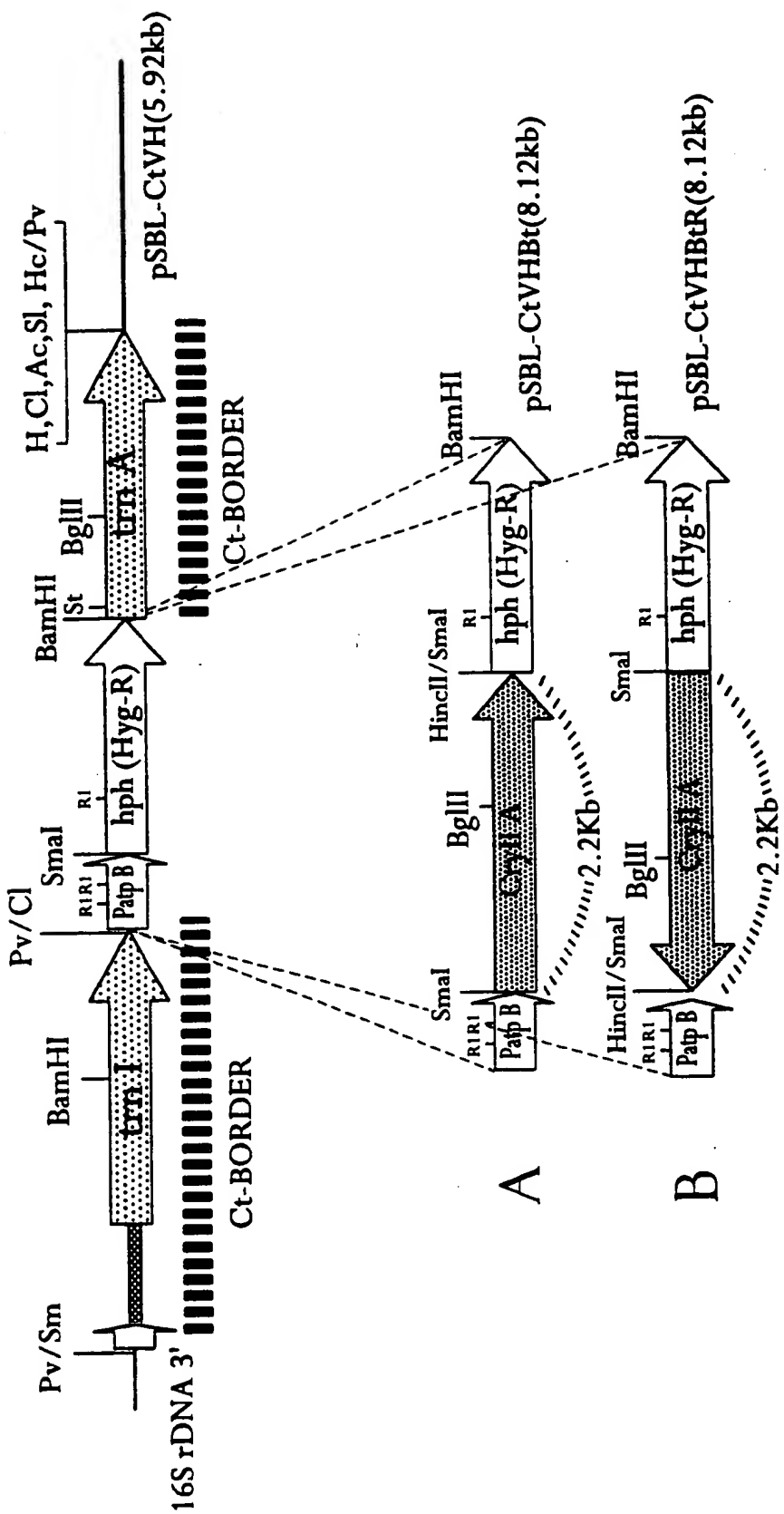


FIG. 8

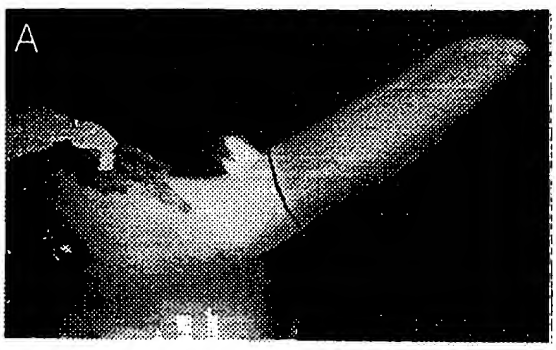


FIG. 10A

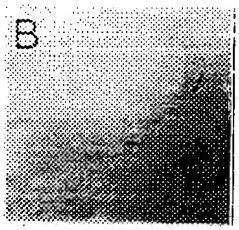


FIG. 10B

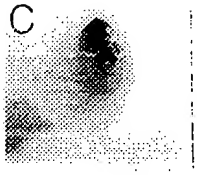


FIG. 10C


FIG. 10D


FIG. 10E


FIG. 10F

FIG. 10G

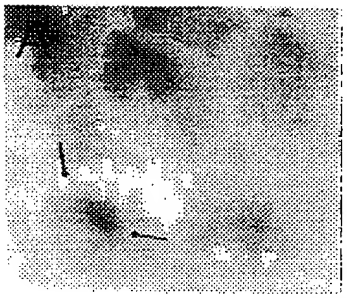

FIG. 12A

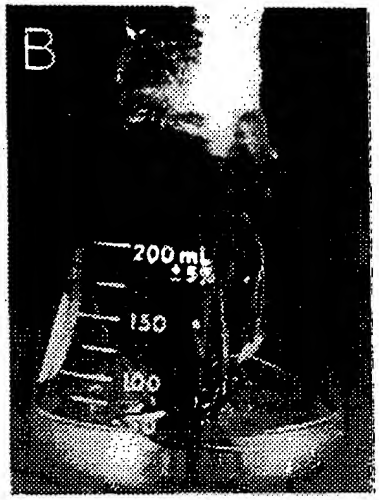


FIG. 12B


FIG. 12C

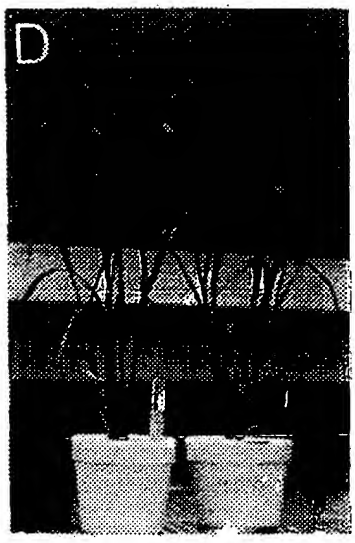


FIG. 12D

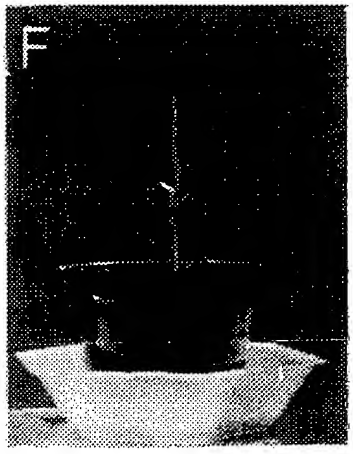


FIG. 12E


FIG. 12F

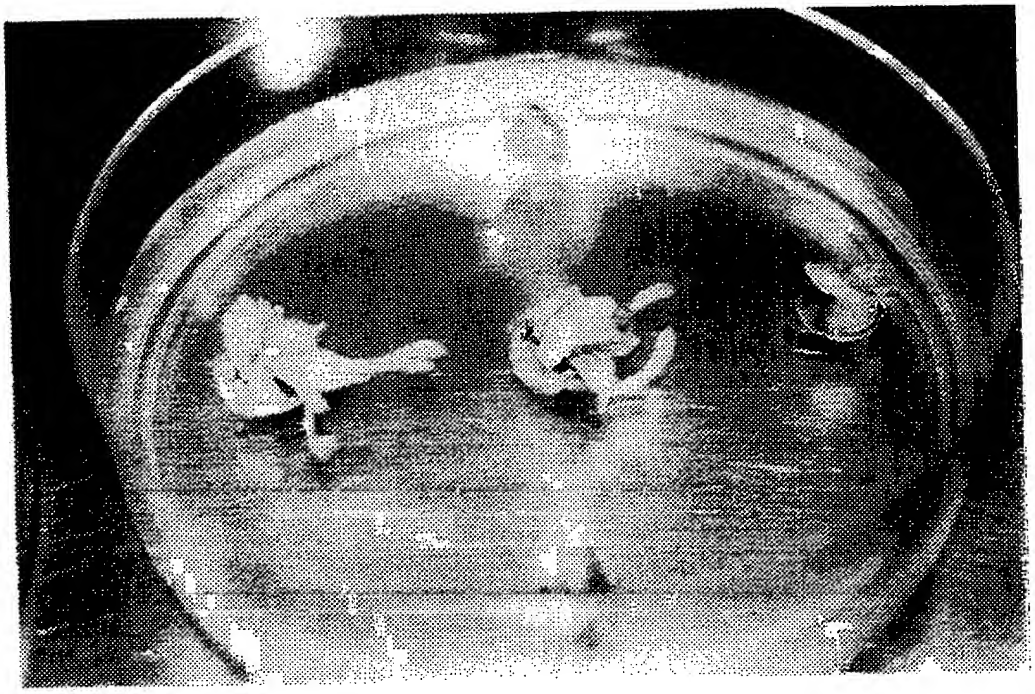

FIG. 14

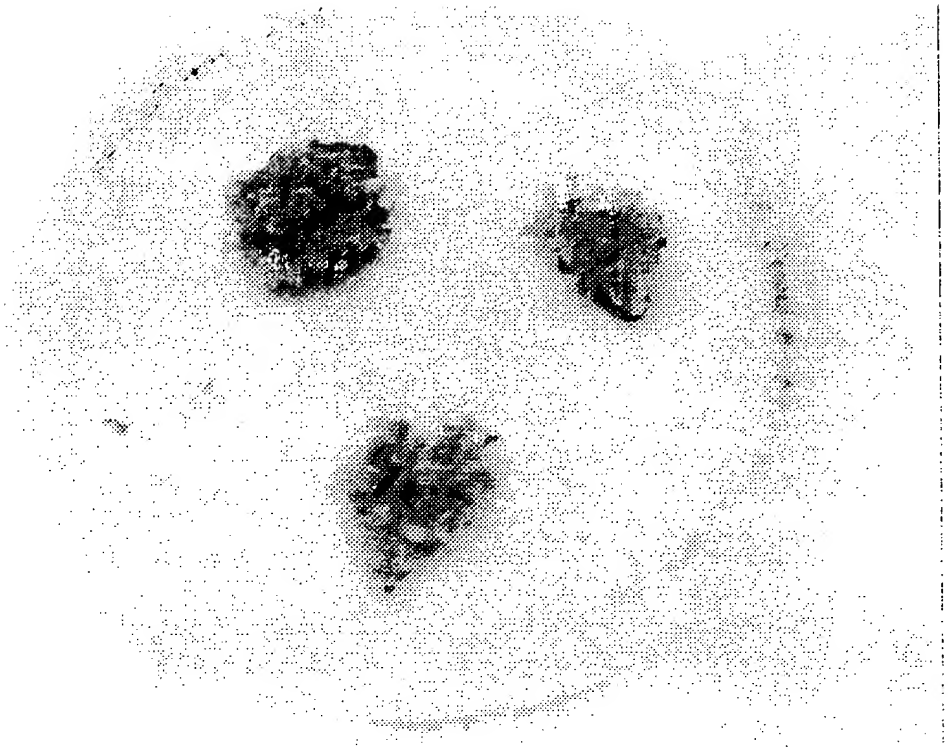

FIG. 16

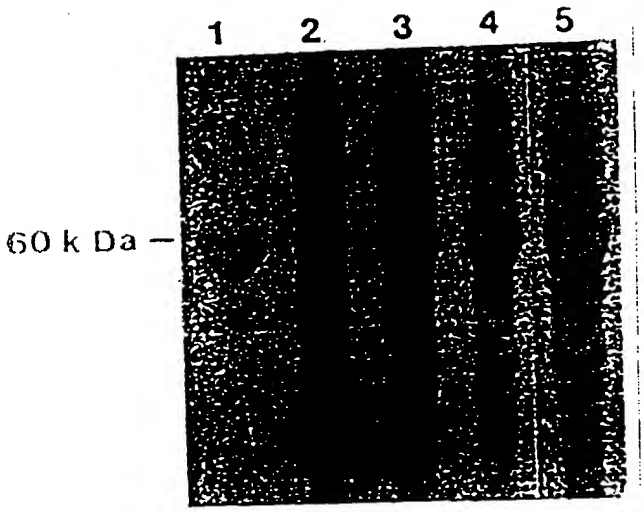


FIG. 18

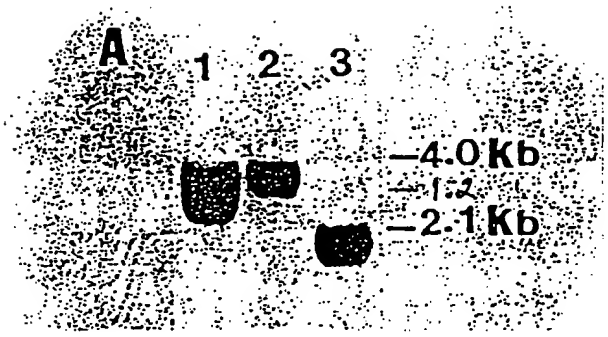


FIG. 20A

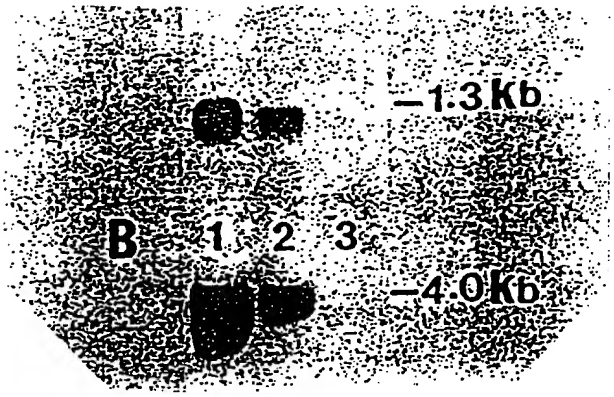


FIG. 20B

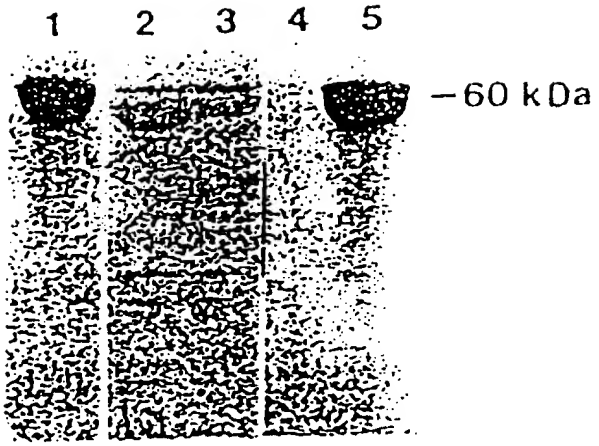


FIG. 22

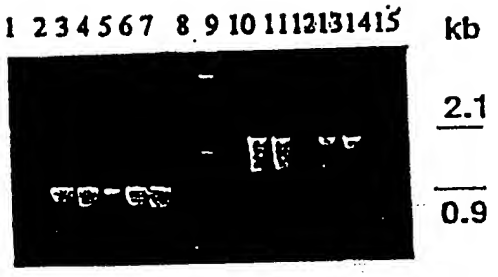


FIG. 24A

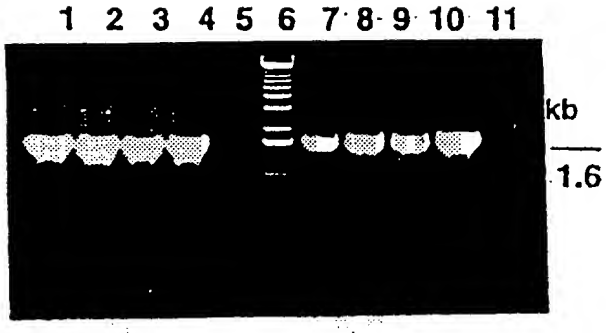


FIG. 24B

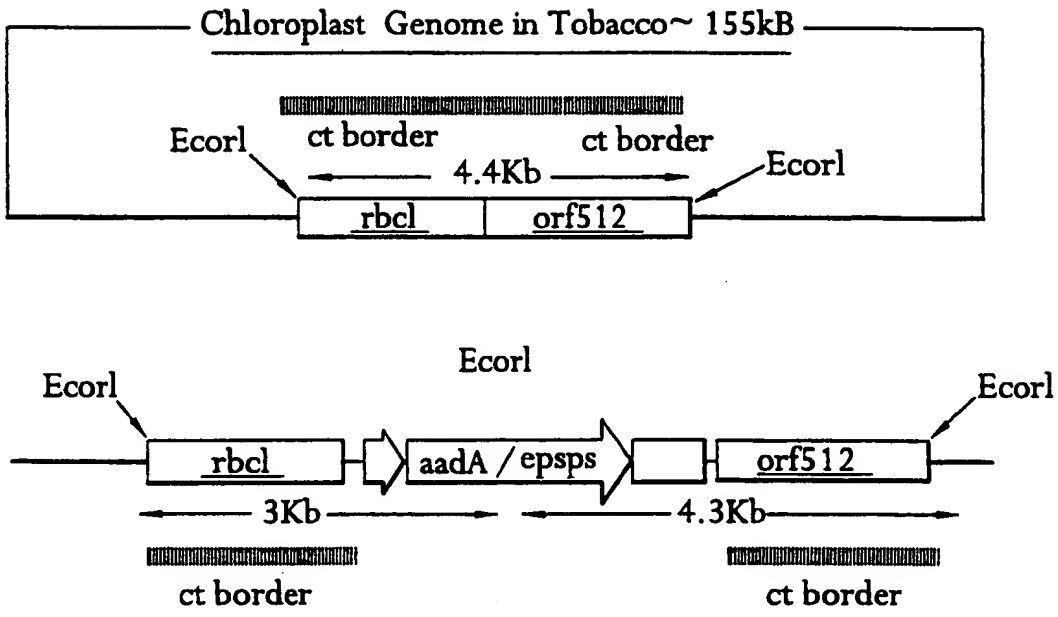


FIG. 25C

FIG. 28A

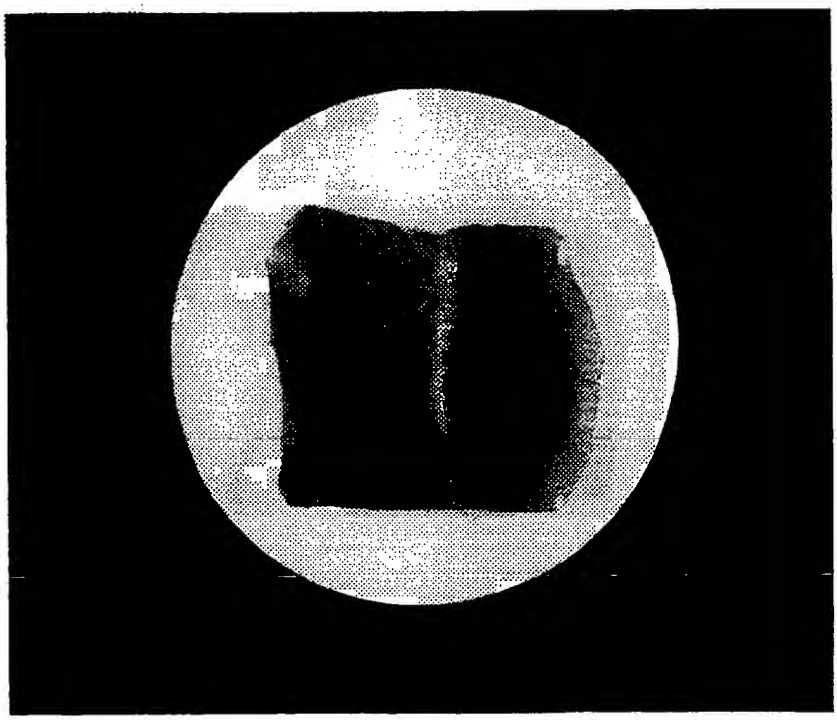

FIG. 28B

# INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/IB 98/01199

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | MCBRIDE, K.E., ET AL. : "amplification of a chimeric Bacillus gene in chloroplasts leads to an extraordinary level of an insecticial protein in tobacco" BIO/TECHNOLOGY, vol. 13, 13 April 1995, pages 362-365, XP002090404 see the whole document --- | 1-3,14, 171,179, 183 |
| X | ZOUBENKO, O.V., ET AL. : "efficient targeting of foreign genes into the tobacco plastid genome" NUCLEIC ACID RESEARCH, vol. 22, no. 19, 1994, pages 3819-3824, XP002090405 see the whole document --- | 1-3,114, 171,179, 183 |

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 19 January 1999 | 01/02/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Holtorf, S |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/IB 98/01199

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claims 28-30 were read as referring to sequences that originate from the same plant species as the target plant.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

What is claimed is:

1. An integration and expression plastid vector competent for stably transforming a plastid genome to confer stress tolerance which comprises an expression cassette which comprises as operably joined components, a 5' part of plastid DNA sequence inclusive of a spacer sequence, a promoter operative in said plastid, an antibiotic-free selectable marker sequence, a DNA sequence encoding an enzyme that catalyzes synthesis of an osmoprotectant, at least one restriction site for the insertion of a heterologous target DNA sequence, a transcription termination region functional in said plastid, and a 3' part of plastid DNA sequence inclusive of a spacer sequence.

2. A vector according to claim 1, wherein said antibiotic-free selectable marker is BADH or the chlB gene.

3. An integration and expression plastid vector competent for stably transforming a plastid genome to confer stress tolerance which comprises an expression cassette which comprises as operably joined components, a 5' part of plastid DNA sequence inclusive of a transcriptionally active spacer sequence, a promoter operative in said plastid, a selectable marker sequence, a DNA sequence encoding an enzyme which catalyzes synthesis of an osmoprotectant, at least one restriction site for the insertion of a heterologous target DNA sequence, a transcription termination region functional in said plastid, and the 3' part of the plastid DNA sequence inclusive of a transcriptionally active spacer sequence.

4. A vector according to any of claims 1 to 3 further comprising a heterologous DNA sequence which codes for a molecule of interest that is inserted in one of the restriction sites.

5. A vector according to claim 4 wherein the molecule of interest is a polypeptide.

6. A vector according to any of claims 1 to 5, wherein said vector further comprises a ribosome binding site and a 5' untranslated region (5' UTR) to enhance expression.

7. A vector according to any claims 1 to 6, wherein the osmoprotectant is selected from a group consisting of sugars, sugar alcohols, sugar derivatives, and amino acids including proline and glycine-betaine.

8. A vector according to any of claims 1 to 7 wherein the osmoprotectant is trehalose.

9. A vector according to any of claims 1 to 8 wherein the enzyme is at least one of the complex TPS1, TPS2, TPS3 or TSL1.

10. The vector according to any of claims 1 to 9 wherein the enzyme is selected from a group consisting of TSP1, *E. Coli* otsA, stachyose, and ononitol.

11. The vector according to any of claims 1 to 10 wherein the osmoprotectant is a sugar

12. The vector according to any claims 1 to 11, wherein the osmoprotectant is a sugar selected amongst monosaccharides preferably fructose, disaccharides preferably sucrose, trisaccharides preferably raffinose and dulcitol.

13 The vector according to any claims 1 to 12 wherein the osmoprotectant is a sugar alcohol.

14 The vector of claim 13 wherein the sugar alcohol is a polyhydric alcohol.

15 The vector of claim 14, wherein the polyhydric alcohol is selected amongst trihydricalcohols preferably glucoglycerol, tetrahydric alcohols preferably erythritol and hexahydric alcohols preferably mannitol or sorbitol.

16 A vector according to any claims 1 to 15, wherein at least one DNA encodes a component of trehalose synthase that is under the control of a promoter to produce a transgenic plant.

17. The vector of claim 16 wherein the promoter is constitutive.

18. The vector of claim 16 wherein the promoter is tissue specific, light-induced, or stress-induced.

19 The vector according to any claims 1 to 18, wherein said plastid is a green plastid.

20. The vector according to any claims 1 to 19 wherein said plastid is a chloroplast.

21. The vector according to any claims 1 to 20 wherein the vector is a universal chloroplast vector.

22. The vector according to any claims 3 to 21, wherein said transcriptionally active spacer sequence comprises a portion of the intergenic spacer two region between and inclusive of the $tRNA^{Ile}$ and the $tRNA^{Ala}$ genes of a chloroplast genome.

23. The vector according to any claims 3 to 22, wherein the spacer region is located in an inverted repeat of the chloroplast genome.

24 The vector according to any claims 1 to 18, wherein said plastid is a non-green plastid.

25 The vector according to any claims 3 to 24, wherein said transcriptionally active spacer sequence is conserved in the plastid genome of different plant species.

26 A stably transformed plant which has been transformed by the vector of any one of claims 1 to 25, wherein the transformed plant is more tolerant of stresses selected from a group consisting of water-deprivation, freezing, salt, heat and cold than is the untransformed plant.

27. The plant according to claim 26 wherein the plant does not include target DNA.

28. A stably transformed plant according to any of claims 26 or 27, or the progeny thereof including seeds, wherein said plant displays no negative pleiotropic effects.

29. A transgenic plant according to any claims 26 to 28, wherein the plant is selected amongst the group consisting of solanaceous plants and crop plants, edible for a mammal, preferably a human.

30 A transgenic plant according to any claims 26 to 29, wherein the plant is selected amongst the group consisting of a monocotyledonous plants, preferably selected from the group of rice, wheat, grass, rye, barley, oat, and maize, and dicotyledonous plants preferably selected from the group of soybean, peanut, grape, sweet potato, pea, canola, tobacco, tomato, cotton, potato, and brassica.

31 A method of conferring drought resistance to plants, said method comprising introducing into the plastid of plant species that are susceptible to water

stress, an expression cassette which comprises as operably joined components, a 5' part of the plastid DNA sequence inclusive of a transcriptionally active spacer sequence, a promoter operative in said plastid, a DNA sequence encoding a yeast gene which confers osmoprotection, a heterologous DNA sequence encoding a molecule of interest, a selectable marker sequence, a transcription termination region functional in said plastid, and a 3' part of the plastid DNA sequence inclusive of a transcriptionally active spacer sequence.

32. The method of claim 31, wherein said method further comprises culturing said plant in a plant growth medium containing an effective amount of polyethylene glycol (PEG) for selection, and selecting transformed plant cells capable of growth in said plant growth medium.

33 The method according claim 32, wherein said method further comprises regenerating the selected transformed plant cells into stable transgenic plants.

34 A method of increasing trehalose accumulation in plant cells thereby conferring osmotic stress resistance to said plant cells, where said method comprises introducing to the plastid of plant species that are susceptible to osmotic stress an expression cassette which comprises as operably joined components, a 5' part of the plastid DNA sequence inclusive of a transcriptionally active spacer sequence, a promoter operative in said plastid, a DNA sequence encoding the Yeast T6P synthase (TSP) gene which confers drought resistance, a heterologous DNA sequence encoding a molecule of interest, a selectable marker sequence, a transcription termination region functional in said plastid, and a 3' part of the plastid DNA sequence inclusive of a transcriptionally active spacer sequence.

35. The method of claim 34, wherein said method further comprises culturing said plant in a plant growth medium containing an effective amount of polyethylene glycol (PEG) for selection, and selecting transformed plant cells capable of growth in said plant growth medium.

36. The method according to claim 35, wherein said method further comprises regenerating the selected transformed plant cells into stable transgenic plants.

37 The method according to any of claims 31 to 36, wherein the plastid is a green plastid preferably a chloroplast.

38. The method according to any of claims 31 to 36, wherein said plastid is a non-green plastid.

39. The method according to any of claims 31 to 38, wherein said transcriptionally active spacer sequence is conserved in the plastid genome of different plant species.

40. The method according to any of claims 31 to 39, wherein said transcriptionally active spacer sequence comprises a portion of the intergenic spacer two region between and inclusive of the tRNA Ala genes of a chloroplast genome.

41. The method according to any of claims 31 to 40, wherein said method confers no pleiotropic effects on said plant.